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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. §371**

MERCK 2289

U.S. APPLICATION NO. (If known, see 37 CFR §1.5)

**09/913494**

INTERNATIONAL APPLICATION NO.

PCT/EP00/00978

INTERNATIONAL FILING DATE

8 FEBRUARY 2000

PRIORITY DATE CLAIMED

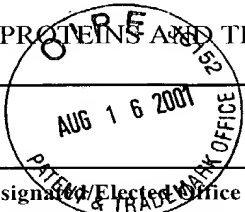
19 FEBRUARY 1999

TITLE OF INVENTION

GLUCOSE DEHYDROGENASE FUSION PROTEINS AND THEIR UTILIZATION IN EXPRESSION SYSTEMS


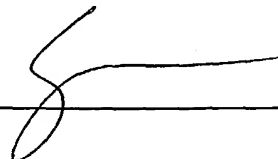
APPLICANT(S) FOR DO/EO/US

LINXWEILER, Winfried, et al .



Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19<sup>th</sup> month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. §371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. §371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).
- Items 11. to 16. below concern document(s) or information included:
  11. ☐ An Information Disclosure Statement under 37 C.F.R. §§1.97 and 1.98.
  12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§3.28 and 3.31 is included.
  13. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
  14. ☐ A substitute specification.
  15. ☐ A change of power of attorney and/or address letter.
  16. ☐ Other items or information:

U.S. APPLICATION NO. (if known, see 37 CFR §1.5) <b>09/913494</b>		INTERNATIONAL APPLICATION NO. PCT/EP00/00978		ATTORNEY'S DOCKET NUMBER MERCK 2289	
17. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR §1.492 (a) (1) - (5)):</b> Search Report has been prepared by the EPO or JPO..... \$860.00 International preliminary examination fee paid to USPTO (37 CFR §1.482)..... \$690.00 No international preliminary examination fee paid to USPTO (37 CFR §1.482) but international search fee paid to USPTO (37 CFR §1.445(a)(2))..... \$710.00 Neither international preliminary examination fee (37 CFR §1.482) nor international search fee (37 CFR §1.445(a)(2)) paid to USPTO..... \$1000.00 International preliminary examination fee paid to USPTO (37 CFR §1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... \$100.00  <div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div>				<b>CALCULATIONS</b> PTO USE ONLY	
				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 C.F.R. §1.492(e)). <input type="checkbox"/> 20 <input type="checkbox"/> 30					
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 - 20 =	0	x \$ 18.00	\$0.00	
Independent claims	1 - 3 =	0	x \$ 80.00	\$0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$ 270.00		
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$860.00	
Reduction of 1/2 for filing by small entity, if applicable. A Verified Small Entity Statement must also be filed (Note 37 C.F.R. §§1.9, 1.27, 1.28).					
<b>SUBTOTAL =</b>				\$860.00	
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 C.F.R. §1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30					
<b>TOTAL NATIONAL FEE =</b>				\$860.00	
Fee for recording the enclosed assignment (37 C.F.R. §1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§3.28, 3.31). \$40.00 per property.					
<b>TOTAL FEES ENCLOSED =</b>				\$860.00	
				Amount to be refunded:	
				charged:	
a. <input checked="" type="checkbox"/> A check in the amount of <u>\$860.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. <u>13-3402</u> in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>13-3402</u> . A duplicate copy of this sheet is enclosed.					
<b>NOTE: Where an appropriate time limit under 37 C.F.R. §§1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. §1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO: Customer Number 23,599					
 <b>23599</b> PATENT TRADEMARK OFFICE			SIGNATURE  _____ NAME <b>Anthony J. Zelano</b> _____ 27,969 REGISTRATION NUMBER		
Filed: 16 August 2001					
AJZ:kmo					

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**IN THE UNITED STATES DESIGNATED/ELECTED OFFICE**

International Application No. : PCT/EP00/00978  
International Filing Date : 8 FEBRUARY 2000  
Priority Date(s) Claimed : 19 FEBRUARY 1999  
Applicant(s) (DO/EO/US) : LINXWEILER, Winfried, et al.  
Title: GLUCOSE DEHYDROGENASE FUSION PROTEINS AND THEIR  
UTILIZATION IN EXPRESSION SYSTEMS

**PRELIMINARY AMENDMENT**

Commissioner for Patents  
Washington, D.C. 20231

SIR:

Prior to calculating the national fee, and prior to examination in the National Phase of the above-identified International application, please amend as follows:

**IN THE CLAIMS:**

4. (Amended) DNA, characterized in that it codes for a fusion protein according to Claim 1.
7. (Amended) Use of glucose dehydrogenase as detector protein for any recombinant protein/polypeptide X in a fusion protein according to Claim 1.
8. (Amended) Use of glucose dehydrogenase in a detection system for the expression of a recombinant protein/polypeptide X as constituent of a fusion protein according to Claim 1.
9. (Amended) Use of glucose dehydrogenase for detecting protein-protein interactions, where one partner corresponds to the recombinant protein/polypeptide X in Claim 1.
10. (Amended) Use of glucose dedydrogenase in a fusion protein according to Claim 1 as detector protein for any third protein/polypeptide which is not a constituent of the

fusion protein according to Claim 1 and is able to bind to the second sequence of the protein/polypeptide X in the said fusion protein.

13. (Amended) Method for the rapid detection of any recombinant protein/polypeptide X by gellectrophoresis, characterized in that a fusion protein according to Claim 1 is prepared and fractionated by gel electrophoresis, and the recombinant protein/polypeptide to be detected in the gel is visualized via the enzymic activity of glucose dehydrogenase.

17. (Amended) Method according to Claim 13, characterized in that the specific staining of the glucose dehydrogenase is followed by a general protein staining.





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PCT/EP00/00978

Glucose dehydrogenase fusion proteins and their use in  
expression systems

5 The invention relates to novel recombinant fusion  
proteins which comprise as one constituent a protein  
sequence having the biological activity of glucose  
dehydrogenase (GlcDH), and to their use for the simple  
and efficient detection of any proteins/polypeptides,  
which preferably serve as fusion partners, and for the  
10 rapid optimization of expression systems which are able  
to express the said proteins/polypeptides.

In this regard, GlcDH or the sequence having the  
biological activity of GlcDH assumes the role of a  
15 marker or detector protein. A particular characteristic  
of this enzyme is exceptional stability to denaturing  
agents such as SDS. GlcDH as marker or detector protein  
shows undiminished enzymatic activity even after the  
reducing and denaturing conditions of SDS-PAGE gels.  
20 Fusion proteins comprising GlcDH can therefore be  
detected using a sensitive enzymatic reaction based on  
this surprising behaviour. It is thus also possible  
with GlcDH as marker for the required expressed protein  
to be detected rapidly, at low cost and efficiently.

25 It is furthermore possible in a number of cases for  
(GlcDH-protein/polypeptide fusion proteins to be  
expressed in higher yield and stability, especially in  
E. coli, than without GlcDH. Corresponding fusion  
30 proteins can thus be used per se for obtaining and  
preparing proteins/polypeptides.

The in vivo expression of recombinant proteins is  
playing an ever increasing part in biotechnology. The  
35 ability to obtain, purify and detect cloned gene  
products from pro- and eukaryotic expression systems  
such as, for example, bacterial, yeast, insect or  
mammalian cells is frequently also used for studies of

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In order to investigate the function of proteins and their interaction partners which are important for the



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function, proteins are usually expressed in eukaryotic cells. The post-transcriptional modifications which are important for the function, and the correct compartmentation can take place therein. In addition,  
5 other proteins important for the correct folding and processing are present.

Eukaryotic expression systems are also appropriate for expressing relatively large proteins and proteins which  
10 require post-transcriptional modifications such as, for example, S-S bridge formation, glycosylation, phosphorylation etc. for correct folding. Since these systems are usually complicated and costly, and the expression rate is below that of E. coli, it is  
15 particularly important to have a detection system which is rapid, reliable, sensitive and reasonably priced.

Numerous gene fusion systems exist for detecting foreign proteins which have been formed by  
20 recombination and whose biological function is unknown. In these, the expressed fusion protein is detected via the fusion protein portion whose function is known.

A sensitive detection system is necessary in order to  
25 determine correct expression, the amount expressed, the molecular weight and the functional activity of the fusion protein formed. The number of proteins of unknown function is increasing rapidly and it is becoming increasingly important to develop rapid and  
30 cost-effective detection systems therefor. With most gene fusion systems, immunological methods such as, for example, the enzym-linked immunosorbent assay (ELISA) or the Western blot are employed, in which fusion proteins formed by recombination are detected with the  
35 aid of specific antibodies.

However, corresponding fusion proteins not only have the described advantage that the foreign protein can easily be detected and analysed indirectly; on the

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contrary in many cases they allow the required protein to be expressed in higher yields than would be the case without its fusion partner. Each fusion partner has advantages, which it is not uncommonly able to transfer  
5 to the other partner, in a particular expression system. Thus, for example, the sensitivity of some proteins to protolytic [sic] degradation can be reduced when it is [sic] in the form of a fusion protein. Fusion proteins also frequently have more favourable  
10 solubility and secretion properties than the individual components.

There are thus numerous reasons for carrying out gene fusions for expressing recombinant proteins in  
15 heterologous hosts. These are: increasing the solubility of foreign proteins, increasing the stability of soluble foreign proteins, localizing the foreign protein in a specific section of the cell, rapid isolation of foreign proteins by simplified  
20 purification strategies, possibility of the fusion protein to be specifically cleaved off, possibility of rapid detection of the foreign protein from unpurified cell extracts.

25 At present there are many functional tests for testing the expression of recombinant proteins with the aid of gene fusion systems. These comprise simple tests which usually make direct detection possible from unpurified cell extracts. However, the test systems differ  
30 considerably in the time taken, throughput and sensitivity.

For the abovementioned purposes it is possible to distinguish two types of fusion proteins. On the one  
35 hand fusion proteins which consist of the required protein and a usually short oligopeptide. This oligopeptide ("tag") functions as a marker or recognition sequence for the required protein. A tag may additionally simplify purification.



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maltose and maltodextrins through the bacterial membrane (Kellermann et al., 1982). It has been used in particular for expressing and purifying alkaline phosphatase on a crosslinked amylose column. The intein system is specifically suitable for rapid purification of a target protein. The intein gene has the sequence for the intein chitin binding domain (CBD), through which the fusion protein can be bound directly from the cell extract onto a chitin column and thus purified (Chong et al., 1997).

Glucose dehydrogenase (GlcDH) is a key enzyme during the early phase of sporulation in *Bacillus megaterium* (Jany et al., 1984). It specifically catalyses the oxidation of  $\beta$ -D-glucose to D-gluconolactone, with  $\text{NAD}^+$  or  $\text{NADP}^+$  acting as coenzyme. Apart from bacterial spores, the enzyme also occurs in the mammalian liver. Two mutually independent glucose dehydrogenase genes (gdh) exist in *B. megaterium* M1286 (Heilmann et al., 1988). GdhA and gdhB differ considerably in nucleotide sequence, whereas GlcDH-A and GlcDH-B have, despite differences in the protein sequence, approximately the same substrate specificity. Further information and the corresponding DNA and amino acid sequences are also to be found, for example, in EP-B 0290 768.

The systems described above for detecting foreign proteins which have been formed by recombination and whose biological function is either unknown or inadequately known are usually complicated and time-consuming. This means that improvement and optimization of the expression conditions often cannot be done quickly or simply enough.

It is therefore a great advance to have developed a fusion protein partner which makes faster detection of the fusion protein possible, and does not have the disadvantages described in the state of the art for comparable systems.

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It has now been found that fusion proteins which comprise GlcDH or a sequence which [lacuna] the biological activity of GlcDH are outstandingly suitable for detecting any required "foreign or target protein" more quickly, simply and thus efficiently than using the state of the art described. This property is based on the surprising finding that GlcDH retains its enzymatic activity under conditions under which other enzymes are inactivated (for example with SDS-PAGE).

The possibility of purifying dehydrogenases on immobilized dyes such as Cibachron Blue 3 G or other NAD-analogous compounds such as aminohexyl-AMP, which are similar, owing to their structure, to the NAD<sup>+</sup> coenzyme and likewise bind to all dehydrogenases, is known.

Thus, as part of a fusion protein, glucose dehydrogenase facilitates, owing to its affinity for the dyes which are, for example, immobilized on a gel and which are commercially available, the purification of the fusion protein in one step. It is furthermore possible to detect GlcDH as constituent of a fusion protein by coupling the enzymatic reaction to a sensitive colour reaction, preferably with iodophenyl-nitrophenyl-phenyltetrazolium salt (INT) or nitro blue tetrazolium salt (NBT) (under the stated conditions), which further simplifies indirect detection of the foreign protein. The method for staining GlcDH as marker enzyme additionally has the advantage that it does not impede the customary staining of proteins using, for example, Coomassie dyes or silver staining in the same gel.

In one embodiment of the present invention, the fusion protein consists of, besides GlcDH and the foreign protein, also a tag peptide which can be used for additional characterization of the proteins bound to the tag peptide. The characterization takes place, for example, via the polyhistidine tag, which is recognized

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as antigen by specific antibodies. Detection of the resulting antigen-antibody complex then takes place, for example, using a peroxidase (POD)-labelled antibody via methods known per se. The bound peroxidase  
5 produces, after addition of an appropriate substrate (for example ECL system, Western Exposure Chemiluminescent Detection System, from Amersham), a chemiluminescent product which can be detected using a film suitable for this purpose. The immunological  
10 detection can, however, also take place by a technique known per se, through a specific antibody tag, for example the myc tag. The polyhistidine tag, alone or in combination with the myc tag, additionally has the advantage that the fusion protein can be purified by  
15 binding to a metal chelate column.

However, the GlcDH fusion protein can also be purified and isolated by affinity chromatography directly on a specific anti-GlcDH antibody which has, for example,  
20 been immobilized on a chromatography gel such as agarose.

Another advantage of the invention is that GlcDH can be expressed in soluble form in high yields, preferably in  
25 *E. coli* by the known expression systems (see above). Thus, recombinant glucose dehydrogenase from *Bacillus megaterium* M1286 has been successfully expressed with high enzymatic activity in *E. coli* (Heilmann 1988). The expression of other eukaryotic genes in *E. coli* is  
30 often limited by the instability of the polypeptide chain in the bacterial host. Incorrect folding may lead to aggregation ("inclusion bodies"), reduced or absent biological activity and proteolytic degradation. A corresponding fusion gene in which the GlcDH gene or a  
35 fragment having the biological activity of GlcDH has been ligated to the gene for the required foreign protein, can now be converted according to the invention into the fusion protein with virtually unchanged expression rate and yield compared with the

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GlcDH gene without fusion partner. This can also take place when expression of the foreign protein on its own is not possible per se or is possible only in reduced yields or only in an incorrectly folded state or only  
5 by use of additional techniques. It is thus possible to obtain the required foreign protein by subsequent elimination of the marker protein GlcDH or of the target protein, for example with endoproteases.

10 An example according to the invention of a target protein which can be expressed successfully as fusion protein together with GlcDH in *E. coli* is tridegin. Tridegin is an extremely effective peptide inhibitor for blood coagulation factor XIIIa and is derived from  
15 the leech *Haementeria ghilianii* (66 AA, 7.6 kD; Finney et al., 1997).

However, there are no restrictions to be mentioned according to the invention in relation to the nature  
20 and the properties of the foreign protein employed.

The invention is not restricted just to the expression of the fusion proteins according to the invention in *E. coli*. On the contrary, such proteins can also be  
25 synthesized advantageously using methods known per se and appropriate stable vector constructs (for example with the aid of the human cytomegalovirus (CMV) promoter) in mammalian, yeast or insect cells with good expression rates.

30

It is accordingly possible from the above description to characterize the invention in summary as follows and as indicated in the claims:

35 The invention thus relates to a recombinant fusion protein consisting of at least a first and second amino acid sequence, the first sequence having the biological activity of glucose dehydrogenase. The invention particularly relates to a corresponding recombinant

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fusion protein in which the said second sequence is any recombinant protein/polypeptide X or represents parts thereof.

5 The fusion proteins according to the invention may additionally comprise recognition sequences, in particular tag sequences. The invention thus relates further to a corresponding fusion protein which may additionally have at least one other tag sequence or  
10 recognition sequence suitable for detection.

The fusion proteins according to the invention have a wide variety of possible uses. In this connection, glucose dehydrogenase with its properties plays the  
15 crucial part. Thus, the invention relates to the use of glucose dehydrogenase as detector protein for any recombinant protein/polypeptide X in one of the said fusion proteins. The invention further relates to the use of glucose dehydrogenase in a detection system for  
20 the expression of a recombinant protein/polypeptide X as constituent of a corresponding fusion protein. The invention further relates to the use of GlcDH for detecting protein-protein interactions, where one partner corresponds to the recombinant  
25 protein/polypeptide X as defined hereinbefore and hereinafter. Finally, GlcDH may serve according to the invention as detector protein for any third protein/polypeptide, which is not a constituent of the fusion protein but is able to bind to the second  
30 sequence of the protein/polypeptide X in the said fusion protein. GlcDH can furthermore be employed as marker protein for a partner in ELISA systems, Western blot and related systems.

35 Since the invention employs recombinant techniques it also, of course, comprises corresponding vectors, host cells and expression systems. The invention relates not only to these vectors and host cells as such but also to the use of corresponding expression vectors in



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optimizing the expression of a recombinant protein/polypeptide X in a recombinant preparation process, and to the use of a corresponding host cell in optimizing the expression of a recombinant  
5 protein/polypeptide X in such a preparation process.

The invention also relates to a method for the rapid detection of any recombinant protein/polypeptide X by gel electrophoresis, in particular SDS-PAGE gel  
10 electrophoresis, where a corresponding fusion protein is prepared and fractionated by gel electrophoresis, and the recombinant protein/polypeptide to be detected is visualized in the gel via the enzymic activity of glucose dehydrogenase.

15 Employed according to the invention in this connection to detect the enzymic activity of glucose dehydrogenase is a colour reaction based on tetrazolium salts, in particular iodophenylnitrophenyl-phenyltetrazolium salt  
20 (INT) or nitro blue tetrazolium salt (NBT), it being possible for a general protein staining according to the state of the art to follow [sic] where appropriate before or after the said colour reaction has taken place.

25 The figures are briefly explained below

Fig. 1: Construction scheme for the vector pAW2. The vector contains the sequence for GlcDH. The complete  
30 sequence is depicted in Seq. Id. No. 1.

Fig. 2: Construction scheme for the vector pAW3.

Fig. 3: Construction scheme for the vector pAW4. The  
35 vector contains the sequence for GlcDH and tridegin. The complete sequence is depicted in Seq. Id. No. 3.

Fig. 4: Staining of GlcDH on an SDS-PAA gel. The staining method is described in detail in the examples.

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1: Rainbow marker; 2: 0.1  $\mu$ g of GlcDH; 3: 0.05  $\mu$ g of GlcDH; 4: 0.001  $\mu$ g of GlcDH; 5: lysate of HC11 cells; 6: prestained SDS marker.

5 Fig. 5: Detection of the expressed GlcDH enzyme (15% SDS-PAA gel, INT stain); 1: Rainbow marker; 2: 0.2  $\mu$ g of native GlcDH; 3: 10  $\mu$ l of cell extract/1 ml of clone 2 suspension; 4: 10  $\mu$ l of cell extract/1 ml of clone 1 suspension; 5: prestained SDS marker; cell extract  
10 volume: 100  $\mu$ l.

Fig. 6: Serial dilutions from pAW2 expression (15% SDS-PAA gel, INT stain); 1: Rainbow marker; 2: 10  $\mu$ l of cell extract/100  $\mu$ l of suspension; 3: 10  $\mu$ l of cell  
15 extract/1:5 dilution; 4: 10  $\mu$ l of cell extract/1:10 dilution; 5: 10  $\mu$ l of cell extract/1:20 dilution; 6: 0.5  $\mu$ g of GlcDH; 7: broad-range SDS marker; 8: prestained SDS marker; cell extract volume: 100  $\mu$ l.

20 Fig. 7: Detection of the expressed tridegin/GlcDH fusion protein (10% SDS-PAA gel, INT/CBB); 1: broad-range SDS marker; 2: 1  $\mu$ g of GlcDH; 3: 0.5  $\mu$ g of GlcDH; 4: 0.1  $\mu$ g of GlcDH; 5: 500  $\mu$ l of cell extract; 6: 200  $\mu$ l of cell extract; 7: 100  $\mu$ l of cell extract; 8: 500  $\mu$ l  
25 of cell extract (pAW2 expression); cell extract volume: 100  $\mu$ l.

Fig. 8: Immunodetection of tridegin/His and tridegin/His/GlcDH fusion protein (from 10% SDS-PAA  
30 gel, ECL detection) and comparison with tridegin/His/GlcDH (10% SDS-PAA gel, INT-CBB stain); 1: broad-range marker; 2: 1 ml of cell extract (pAW2 expression); 3: 100  $\mu$ l of cell extract (pST106 expression); 4: 200  $\mu$ l of cell extract (pST106  
35 expression); 5: 300  $\mu$ l of cell extract (pAW4 expression); 6: 2.5  $\mu$ g of calin-His positive control; 7: broad-range marker; 8: 100  $\mu$ l [lacuna] (pAW4 expression); cell extract volume: 100  $\mu$ l.

Fig. 9: SDS gel which explains the sensitivity of the detection of GlcDH. 1, 5, 10, 25 and 50 ng of GlcDH and molecular weight markers (left-hand column) are plotted.

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The abbreviations used hereinbefore and hereinafter are explained below

	A	adenine	
	A <sub>x</sub>	absorption at x nm	
10	Ab	antibody	
	Amp	ampicillin	
	AP	alkaline phosphatase	
	APS	ammonium peroxodisulphate	
	AA	amino acid	
15	bla	β-lactamase gene	
	BIS	N,N'-methylenebisacrylamide	
	bp	base pairs	
	BSA	bovine serum albumin	
	C	cytosine	
20	cDNA	copy (complementary) DNA	
	CBB	Coomassie Brilliant Blue	
	CIP	calf intestinal phosphatase	
	dNTP	2'-deoxyribonucleoside [sic] 5'-triphosphate	
	ddNTP	2',3'-deoxyribonucleoside [sic] 5'-triphosphate	
25	DMF	dimethylformamide	
	DMSO	dimethyl sulfoxide	
	DNA	deoxyribonucleic acid	
	dsDNA	double-stranded DNA	
	DTT	dithiothreitol	
30	ECL	Exposure <sup>TM</sup> Chemiluminescence	
	EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid, disodium salt	
	ELISA	enzyme-linked immunosorbent assay	
	EtBr	ethidium bromide	
35	EtOH	ethanol	
	f.c.	final concentration	
	FACS	fluorescent-activated [sic] cell sorting	
	G	guanine	
	GFP	green fluorescent protein	

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	GlcDH	glucose dehydrogenase (protein)
	gdh	glucose dehydrogenase (gene)
	GST	glutathione S-transferase
	His	histidine residue
5	HRP	horseradish peroxidase
	IB	inclusion body
	IgG	immunoglobulin G
	INT	iodonitrotetrazolium violet
	kb	kilobase pairs
10	kD	kilodalton
	mA	milliampere
	m-RNA	messenger RNA
	MBP	maltose-binding protein
	MCS	multiple cloning site
15	M <sub>r</sub>	relative molecular weight
	NAD(P)	nicotinamide adenine dinucleotide (phosphate), free acid
	Od <sub>x</sub>	optical density at x nm
	ompA	outer membrane protein A
20	ori	origin of replication
	PAA	polyacrylamide
	PAGE	polyacrylamide gel electrophoresis
	PCR	polymerase chain reaction
	POD	peroxidase
25	PVDF	polyvinylidene difluoride
	RNA	ribonucleic acid
	RNase	ribonuclease
	rpm	revolutions per minute
	rRNA	ribosomal RNA
30	RT	room temperature
	SDS	sodium dodecyl sulfate
	ssDNA	single-stranded DNA
	Strep	streptavidin
	T	thymine
35	T <sub>m</sub>	melting point (DNA duplex)
	t-RNA	transfer RNA
	Taq	<i>Thermophilus [sic] aquaticus</i>
	TCA	trichloroacetic acid
	TEMED	N,N,N',N'-tetramethylethylenediamine

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Tet        tetracycline  
 Tris      tris(hydroxymethyl)aminomethane  
 U         unit of enzymic activity  
 U         uracil  
 5 UV       ultraviolet radiation  
 ON        overnight  
 V         volt  
 VIS       visible  
 w/v      weight per volume

10

References:

- Aoki et al. (1996), FEBS Letters **384**, 193-197  
 Banauch et al. (1975), Z. Klin. Chem. Klin. Biochem.  
 15        Vol. **13.**, 101-107  
 Bertram, & Gassen (1991) Gentechnische Methoden,  
       Eine Sammlung von Arbeitsanleitungen für das  
       molekularbiologische Labor. Gustav Fischer Verlag,  
       Stuttgart, Jena, New York  
 20    Brewer & Sassenfeld (1985), Trends in Biotechnology  
       **3**, No. 5, 119-122  
 Brown, T.A. (1993) Gentechnologie für Einsteiger:  
       Grundlagen, Methoden, Anwendungen. Spektrum  
       Akademischer Verlag, Heidelberg; Berlin; Oxford  
 25    Casadaban et al. (1990), Methods in Enzymology **100**, 293  
       Chalfie et al. (1994), Science **263**, 802-805  
       Chong, S. et al. (1997), Gene **192**, 271-281  
       Collins-Racie et al. (1995), Biotechnology **13**, 982-987  
       Di Guan et al. (1988), Gene **67**, 21-30  
 30    Ettinger et al. (1996), Proc. Natl. Acad. Sci. USA **93**,  
       13102-13107  
       Finney et al. (1997), Biochem. J. **324**, 797-805  
       Gazitt et al. (1992), Journal of Immunological Methods  
       **148**, 159-169  
 35    Ghosh et al. (1995), Analytical Biochemistry **225**, 376-  
       378  
       Goeddel et al. (1979), Proc. Natl. Acad. Sci. U.S.A.  
       **76**, 106-110

- Hafner & Hoff (1984), Genetik. Neubearbeitung, Schrödel-Verlag, Hanover
- Harlow & Lane (1988), A Laboratory Manual, Cold Spring Harbor
- 5 Harris & Angal (1990) Protein purification applications: a practical approach. Oxford University Press, Oxford; New York; Tokyo
- Heilmann et al. (1988), Eur. J. Biochem. **174**, 485-490
- Hilt et al. (1991), Biochimica et Biophysica Acta **1076**,  
10 298-304
- Ibelgaufits, H. (1990) Gentechnologie von A bis Z. Erweiterte Ausgabe, VCH-Verlag, Weinheim
- Inouye et al. (1994), FEBS Letters **341**, 277-280
- Itakura et al. (1977). Science **198**, 1056-1063
- 15 Jany et al. (1984), FEBS Letters **165**, no. 1, 6-10
- Kellermann & Ferenci (1982), Methods in Enzymology **90**,  
459-463
- Laemmli (1970), Nature **227**, 680-685
- La Vallie & McCoy, (1995), Curr. Opin. Biotechnol. **6**,  
20 501-506
- Makino et al. (1989), Journal of Biological Chemistry **264**, No. 11, 6381-6385
- Marston (1986), Biochem. J. **240**, 1-12
- Moks et al. (1987), Biochemistry **26**, 5239-5244
- 25 Okorokov et al. (1995), Protein Expr. Purif., **6**, 472-480
- Pharmacia Biotech 1: From Cells to Sequences, A Guide to PCR analysis of nucleic acids
- Pharmacia Biotech 2: The Recombinant Protein Handbook,  
30 Principles and Methods
- Pitzurra et al. (1990), Journal of Immunological Methods **135**, 71-75
- Pogge v. Strandmann et al. (1995), Protein Engineering **8**. No. 7, 733-735
- 35 Sambrook et al., (1989) Molecular Cloning, A Laboratory Manual 1, Second Edition, Cold Spring Harbor Laboratory Press, USA
- Sanger et al. (1977), Proc. Natl. Acad. Sci. U.S.A. **74**,  
5463-5467

- 17 -

Schein (1989), Bio/Technology 7, 1141-1149

Scopes (1994) Protein purification; principles and practice, 3<sup>rd</sup> ed., Springer-Verlag, New York; Berlin; Heidelberg

5 Smith & Johnson (1988), Gene 67, 31-40

Smith et al. (1988), Journal of Biological Chemistry 263, No. 15, 7211-7215

Uhlén & Moks (1990), Gene Fusions for Purpose of Expression: An Introduction [12]. Methods in Enzymology 185, 129-143

10

Uhlén et al. (1983), Gene 23, 369

Unless specified otherwise, the methods and techniques used for this invention correspond to methods and processes sufficiently well known and described in the relevant literature. In particular, the disclosure contents of the abovementioned publications and patent applications, especially by Sambrook et al. and Harlow & Lane, and EP-B-0290 768, are comprised in the invention. The plasmids and host cells used according to the invention are as a rule exemplary and can in principle be replaced by vector constructs which are modified or have a different structure, or other host cells as long as they still have the constituents stated to be essential to the invention. The preparation of such vector constructs, and the transfection of appropriate host cells and the expression and purification of the required proteins correspond to standard techniques which are substantially well known and may likewise be modified according to the invention within wide limits.

25

30

The invention is described further below. Further details are explained in the examples.

35

The *Bacillus megaterium* GlcDH structural gene was modified by PCR with the plasmid pJH115 (EP 0290 768) acting as template. The amplified fragment (0.8 kb), which had a PstI recognition sequence at one end and an

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Eco47III recognition sequence at the other, was digested with these enzymes and cloned into the cytoplasmic (pRG45) or periplasmic (pST84) *E. coli* expression vector (Figs. 1, 2). The resulting plasmids, pAW2 and pAW3, now had a GlcDH gene which encodes a protein of about 30 kD (261 AA) and is located downstream of the strong Tet promoter. The cytoplasmic pAW2 expression vector has a size of about 4 kb. The periplasmic pAW3 secretion vector is slightly larger and differs from pAW2 only by an omp A signal sequence which is upstream of the multiple cloning site (MCS) and makes it possible for the recombinant protein to be secreted into the periplasm. Both vectors additionally have an MCS with 12 different restriction cleavage sites which make in-frame cloning with the following His tag possible. The polyhistidine (6His) tag makes it possible for the recombinant protein to be purified on a metal affinity column. The vector pAW4 finally comprises the tridegin gene and the GlcDH gene, which were connected together by an MCS, and the polyhistidine (6His) tag which is ligated downstream to the GlcDH gene. The individual constructs are depicted in Figs. 1, 2 and 3. However, the chosen plasmid constructs are only by way of example and do not restrict the invention. They may be replaced by other suitable constructs containing the DNA sequences mentioned. The preparation of the vectors and the clones and expression of the proteins are specified further in the examples.

The sensitivity of the activity staining was carried out [sic] for native GlcDH in a reduced SDS gel. For this purpose, serial concentrations were prepared with native GlcDH ( $c = 1 \text{ mg/ml}$ ;  $A = 200 \text{ U/ml}$ ), and a negative control was prepared. SDS-PAGE and activity staining using INT resulted in the SDS gel depicted in Fig. 3. It was possible with the test employed to detect GlcDH down to a concentration of 50 ng. The



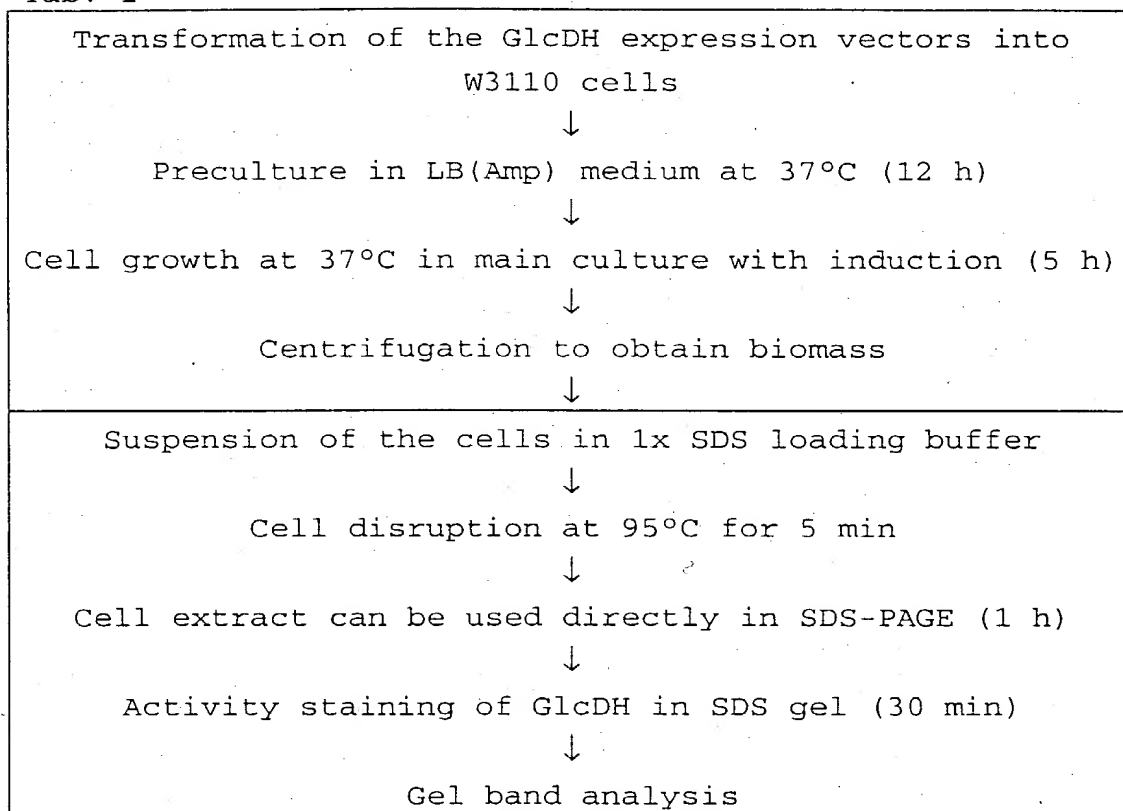
- 19 -

negative control, which contains no GlcDH, shows no band, as expected.

5 The exact molecular weight of the native GlcDH can be determined using marker proteins and with the aid of a calibration plot. To do this, the relative migration distances of the marker proteins were determined and plotted against their respective logarithmic molecular weights.

10 A procedure for the expressions carried out was as depicted in the scheme (Tab. 1):

Tab. 1



15 The plasmid pAW2/clone 9 (pAW2/K9) was transformed into the competent *E. coli* expression strain W3110, and two clones from the resulting transformation plate were used to inoculate a 5 ml preculture. Induction with anhydrotetracycline took place 2 h after inoculation of

20 the main culture. Expression overall lasted 5 h and was stopped at an OD of 1.65 for clone 1 and 1.63 for clone

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2. After SDS-PAGE and GlcDH activity staining, a strong GlcDH band (about 35 kD) was detectable for each clone from 1 ml of cell suspension.

No difference between the resulting GlcDH bands became  
5 evident when SDS-PAGE was carried out under reduced and non-reduced conditions. For this purpose, in each case 500 to 100  $\mu$ l of the cell suspension were investigated in the SDS gel by GlcDH activity staining with INT.

10 In order to illustrate the sensitivity of the GlcDH activity staining compared with Coomassie staining, samples of 100  $\mu$ l of cell suspension, and 1/5, 1/10 and 1/20 dilutions of the cell suspension were prepared. The final volume of the dilutions was likewise 100  $\mu$ l.  
15 The resulting SDS gel was used, after the GlcDH activity staining, for a Coomassie staining to visualize further protein bands. The SDS gel resulting from this is depicted in Figure 4. A distinct band is still evident at the 1/20 dilution using the GlcDH  
20 activity staining, whereas Coomassie-stained bands are now scarcely detectable.

The *Haementeria ghilianii* tridegin structural gene with coupled His tag was modified by PCR with the plasmid  
25 pST106 acting as template. The amplified fragment (0.25 kb), which is flanked by a ClaI recognition sequence and a PstI recognition sequence, was digested with these enzymes and cloned into the cytoplasmic *E. coli* GlcDH fusion vector pAW2. The resulting plasmid  
30 pAW4 now had a tridegin-His-GlcDH fusion protein gene which codes for a protein of about 44 kD and is located downstream of the strong Tet promoter. The cell extract from the *E. coli* strain W 3110 which comprises the cytoplasmic pAW4 plasmid was analysed by SDS-PAGE and  
35 GlcDH activity staining. It was possible therewith to detect several bands stained red-violet at 35, 37, 40 and 43 kD. The 43 kD band comprised the required tridegin-His-GlcDH fusion protein, although its molecular weight was somewhat less than the theoretical

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value of 44 kD. The remaining detectable bands were presumably produced by proteolytic degradation of the fusion protein in *E. coli* since the smallest stained band of 35 kD approximately corresponds to the size of GlcDH. It was possible on the basis of a size comparison to identify the 35 kD band which was formed as the His-GlcDH degradation product.

Carrying out [sic] expression kinetics revealed that proteolytic degradation of the formed fusion protein started 2 hours after induction of the Tet promoter with anhydrotetracycline, that is to say after this time additional bands were detectable in the SDS gel by activity staining. The formed fusion protein was not stable to *E. coli* proteases, which is shown by its relatively fast protein degradation. It was possible, by using the constructed periplasmic GlcDH fusion vector pAW3 to avoid proteolytic degradation of the fusion protein in the cell, because in this case the expressed fusion protein would be secreted into the periplasmic space between *E. coli* cells. *E. Coli* proteases are found mainly in the cytoplasm.

The sensitivity and specificity of the GlcDH fusion protein detection makes it possible for recombinant foreign proteins to be screened rapidly and simply. Sensitivity of the GlcDH detection system was determined using native GlcDH. Detection of native GlcDH activity resulted in a band stained red-violet at about 30-35 kD in the SDS-PAA gel.

Cytoplasmic expression in the *E. coli* strain W 3110 of the recombinant GlcDH from pAW2 showed the same molecular weight. Sensitivity comparison between native GlcDH and recombinant GlcDH was possible by comparing the band intensities.

The developed test system (see examples) additionally makes it possible to carry out double staining of the SDS gels. In the first staining there is specific detection of the GlcDH bands. The background staining can be followed by a conventional protein staining, for

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example a Coomassie staining of the remaining proteins. GlcDH surprisingly retains according to the invention under reducing conditions in the presence of SDS its complete activity, which makes rapid detection in the  
5 SDS gel possible.

It is furthermore possible according to the invention to increase the sensitivity of the detection of GlcDH activity by using nitro blue tetrazolium salt (NBT) as  
10 substrate for GlcDH. The reaction rate for the GlcDH detection using INT can, however, be increased further by using Triton X-100 (1% final solution) or adding NaCl (1 M final solution).

15 The recombinant fusion proteins tridegin/His and tridegin/His/GlcDH were obtained by expression of the pST106 and pAW4 plasmids (Figs. 1, 2). After disruption of the cells in the relevant expression mixture, the samples were fractionated by SDS-PAGE and transferred  
20 to a membrane. The tridegin-His-GlcDH fusion protein was detectable immunologically via the His tag present therein by using an anti-<sup>RGS</sup>His antibody in a Western blot. The controls used were purified recombinant calin (leech protein) which has a terminal His tag, and the  
25 cell extract of the expressed recombinant GlcDH which has no His tag. The anti-<sup>RGS</sup>His antibody was able to detect a band at about 37 kD and another band at about 43 kD for the recombinant tridegin/His/GlcDH fusion protein (Fig. 6). Comparison of the sizes of the bands  
30 obtained with the bands obtained after activity staining in the SDS gel shows that the 43 kD band represents the tridegin-His-GlcDH fusion protein and the 37 kD band represents the His-GlcDH degradation product of the complete fusion protein. The calin/His  
35 tag protein produced a band at about 26 kD. The somewhat smaller recombinant tridegin/His tag protein produced a band at about 23 kD plus further bands indicating binding of the His antibody to other expressed proteins. The immunological detection with

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the anti-<sup>RGS</sup>His antibody thus proves that the protein detected at 43 kD and that detected at 37 kD contained a His tag. In addition, the size of the latter protein approximately corresponded to the theoretical size (36.5 kD) of the GlcDH protein with coupled His tag.

In addition to the detection of expression of the recombinant tridegin, the biological activity of tridegin as constituent of the tridegin-GlcDH fusion protein was investigated, in the specific case from pAW4. This test is based on the inhibition of factor XIIIa by native leech gland homogenate and purified tridegin (Finney, et al., 1997). The modified test is described in the examples. As a control, the corresponding fusion protein from pST106 and the GlcDH protein from pAW2 were expressed. Comparison of the enzymic activity with recombinant tridegin expressed either as GlcDH-tridegin fusion protein or as tridegin-His tag in *E. coli* revealed negligible differences. In addition, the recombinant tridegin proteins from the two different expressions showed comparable biological activities to the native leech gland homogenate. It can be concluded from this that fusion with GlcDH has no interfering effect at all on the biological activity of the coexpressed foreign gene.

Tridegin itself (that is to say not as fusion protein) has no activity after *E. coli* expression and is formed as inclusion body. Expression of GlcDH in *E. coli* results in an enzyme with high specific activity and stability in soluble form. It was demonstrated in expression experiments that proteins which have a high solubility capacity on expression in *E. coli* increase the solubility capacity of foreign protein expression when they are fused to the latter (LaVallie, 1995). Fusion of tridegin to GlcDH in this case also increased the solubility of tridegin because it was possible by a biological detection in which tridegin inhibits factor XIIIa to detect the activity of tridegin after *E. coli*

expression as tridegin-His-GlcDH fusion protein. The GlcDH fusion protein is expressed in high yield in *E. coli*.

5 The possibility of expressing cloned genes as fusion proteins containing a protein of known size and biological function markedly simplifies the detection of the gene product. For this reason, as mentioned in the introduction, numerous fusion expression systems have been developed with various detection strategies.

10

A comparison of the known systems with the GlcDH fusion system according to the invention in *E. coli* is shown in Tab. 2. In some systems, the N-terminal fusion protein can be cleaved off from the C-terminal target or foreign protein (Collins-Racie et al., 1995).

15

Tab. 2:

Tag/fusion partner	MW (kD)	Detection	Advantage
GlcDH	30	Function test in the SDS gel	Rapid and low-cost, direct detection in the SDS gel
His tag (Pogge v. Strandmann et al., 1995)	1-7	Western blot, ELISA	Small
Strep-tag (Uhlén et al., 1990)	13	Western blot,	Small
myc epitope (Pitzurra et al., 1990; Gazitt et al., 1992)	1-2	Western blot, ELISA	Small
IgG portions, Fc (Moks et al., 1987; Ettinger et al., 1996)	2-5	Western blot, ELISA	Small, selection of cells (FACS)
GFP (Chalfie et	27	Fluorescence,	Selection of

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al., 1994; Inouye et al., 1994)		Western blot	cells even in the culture dish, several detectable simultaneously (FACS)
Intein (Chong et al., 1997)	48	Western blot	Fusion partner can be deleted
GST (Smith, Johnson, 1988; Gosh et al., 1995)	26	Western blot, colorimetric detection in solution	Fusion partner can be deleted
MBP (Chu di Guan et al., 1988; Kellermann et al., 1982)	40	Western blot	Fusion partner can be deleted

Method	Pre-condition	Time taken	Throughput	Sensitivity	Information
GlcDH detection	GlcDH functionally active	about 3 h	moderate-high	50 ng	protein amount + protein size
ELISA	2 anti-bodies	about 1 day	high	pg-ng	protein amount
Western blot	1-2 anti-bodies Tag on the protein	1-2 days	low	ng	protein size + protein amount

A very great advantage of the GlcDH detection system according to the invention is the fact that it does not require, such as, for example, for the Western blot detection, any antibodies or other materials such as, for example, membranes, blot apparatus, developer machine with films, microtitre plates, titre plate reader etc. This means that the detection of

recombinant fusion proteins using the GlcDH system takes place very much more favourably and rapidly. It is possible with the aid of GlcDH detection to establish not only information about the amount of the expressed fusion protein but also the corresponding size of the fusion protein directly in the SDS-PAA gel without transfer to a membrane. If GlcDH activity is detectable in the fusion protein, the fusion partner ought also as a rule to be functionally active. GlcDH does not interfere with the folding of the fusion partner. The advantages of the GlcDH fusion protein system according to the invention are shown in a comparison hereinafter (Tab. 3 below) by selecting from the literature an efficient method for isolating and detecting a fusion protein obtained in *E. coli*.

The GlcDH fusion protein system according to the invention is furthermore particularly suitable for increasing the solubility of proteins which are formed, especially in *E. coli*, as inclusion bodies and therefore make subsequent protein purification difficult and costly. It is normally necessary to convert proteins formed as inclusion bodies into their native state by elaborate methods. This is unnecessary on use of the fusion proteins according to the invention.

In summary, the advantages of the fusion proteins according to the invention which are in use as GlcDH detection system are as follows.

- Stability under SDS and reducing (denaturing) conditions
- Sensitive GlcDH-specific enzymatic colour test
- Sensitivity as far as at least 50 ng
- Rapid detection directly in the SDS gel with determination of the molecular weight of the fusion partner
- Possibility of additional protein stainings
- Low-cost materials, little expenditure on apparatus



- Good expression in E. coli, including that of the target protein with retention of the biological activity
- Possibility of avoiding inclusion bodies of the foreign/target protein or other aggregates produced by incorrect folding
- Possibility of purifying the fusion protein via affinity chromatography, for example on dyes (Cibacron Blue 3G)

10

Tab. 3

Construction/transformation of the <u>protein A/GFP fusion vector</u>	Construction/transformation of the <u>GlcDH/tridegin fusion vector</u>
↓	↓
Growth of the cells on LB agar plates at 37°C (1 day)	Preculture in LB (Amp) medium at 37°C (12 h)
↓	↓
Cell growth at 25°C (3 days)	Cell growth at 37°C in main culture with induction (5 h)
↓	↓
Suspension of the cells in buffer (pH 8.0)	Suspension of the cells in SDS loading buffer
↓	↓
Cell disruption and removal of cell detritus by centrifugation	SDS cell disruption at 95°C for 5 min
↓	↓
SDS-PAGE for protein separation (1 h)	SDS-PAGE (1 h) with cell extract
↓	↓
Protein transfer to nitrocellulose membrane (1 h)	
↓	
Blocking reaction (1 h)	GlcDH activity staining in

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<p style="text-align: center;">↓</p> <p style="text-align: center;">Antibody reaction (1 h)</p> <p style="text-align: center;">↓</p> <p style="text-align: center;">Incubation in protein A-GFP working buffer (20 min)</p> <p style="text-align: center;">↓</p> <p style="text-align: center;">UV radiation (365 nm)/analysis of the blot</p>	<p style="text-align: center;">the SDS gel (30 min)</p> <p style="text-align: center;">↓</p> <p style="text-align: center;">Analysis of the SDS gel with <u>determination of the</u> <u>molecular weight</u></p>
--	--

The following examples illustrate the invention further without restricting it.

5 **Example 1:**

Primer	Sequence	Length	Use
GlcDH#1	5'- GCGCGAATTCATGTATA CAGATTTAAAAGAT- 3'	32 bases	PCR primer (attaches to the 5' end of gdh and introduces an EcoRI cleavage site)
GlcDH#2	5'- GCGCTTCGAACTATTAG CCTCTTCCTGCTTG-3'	31 bases	PCR primer (attaches to the 3' end of gdh and introduces an SfuI cleavage site)
GlcDH#3	5'- GCGCCTGCAGATGTATA CAGATTTAAAAGAT-3'	31 bases	PCR primer (attaches to the 5' end of gdh and introduces a PstI cleavage site)
GlcDH#4	5'- GCGCAGCGCTCTATTAG CCTCTTCCTGCTTG-3'	31 bases	PCR primer (attaches to the 3' end of gdh and introduces an Eco47III cleavage site)

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Tridegin #1	5'- GCGCATCGATATGAAAC TATTGCCTTGCAAA-3'	31 bases	PCR primer (attaches to the 5' end of tridegin and introduces a ClaI cleavage site)
Tridegin #2	5'- GCGCCTGCAGGTGATGG TGATGCTGATGCGA-3'	31 bases	PCR primer (attaches to the 3' end of tridegin and introduces a PstI cleavage site)
pASK 75 UPN	5'- CCATCGAATGGCCAGAT GATTA-3'	22 bases	Sequencing primer (IRD 41 labelled at the 5' end, attaches in tet p/o of pRG 45 and pST84)
PASK 75 RPN	5'- TAGCGGTAAACGGCAGA CAAA-3'	21 bases	Sequencing primer (5' IRD 41 labelled, attaches in lpp of pRG 45 and pST84)
T7 Seq.s	5'- TAATACGACTCACTATA GGG-3'	20 bases	Sequencing primer (5' IRD 41 labelled, attaches to the T7 priming site of pcDNA3.1/myc-His A, B, C)
Rev Seq.as	5'- TAGAAGGCACAGTCGAG G-3'	18 bases	Sequencing primer (5' IRD 41 labelled, attaches to the BGH reverse priming site of pcDNA3.1/myc-His A, B, C)

The above nucleotides were used according to the invention (Tab. 4).

- 5 Table 5 below summarizes the microorganisms used. All the microorganisms are derived from *E. coli* K12 and belong to risk group 1.

Tab. 5

Strain	Genus/ species	Genotype	Literature
Top10F' One Shot™ Cells	<i>E. coli</i>	F' (lacI <sup>q</sup> Tn10(Tet <sup>R</sup> )) mcrA Δ(mrr-hsdRMS- mcrBC) Φ80lacZΔM15ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str <sup>R</sup> ) endA1 nupG	Top10F' OneShot™ Kit from Invitrogen®
Epicurian Coli®XL1- Blue MRF' Cells	<i>E. coli</i>	Δ(mcrA)183 Δ(mcrCB- hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac(F' proAB lacI <sup>q</sup> ZΔM15Tn10(Tet <sup>i</sup> ))	Stratagene's Competent Cells
TOP10 OneShot™ Cells	<i>E. coli</i>	F' mcrA Δ(mrr-hsdRMS- mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str <sup>R</sup> ) endA1 nupG	TOPO TA Cloning® Kit (Version C) from Invitrogen®
W 3110	<i>E. coli</i>	F' λ <sup>-</sup> WT <i>E. coli</i>	B. Bachmann, Bacteriol. Rev. 36(72) 525-557

Donor organism: M 7037 expression strain (*E. coli* N  
5 4830/pJH 115) of 21.10.96 (supplied by Merck).

pJH 115: pUC derivative, 5.9 kb, O<sub>L</sub>P<sub>L</sub> promoter, gdh, to  
10 (terminator), galK (galactosidase gene), bla (β-  
lactamase gene), ori (origin of replication), 2  
HindIII, 2 BamHI and one each EcoRI and ClaI cleavage  
site.

#### Example 2:

Transformation of plasmids into competent *E. coli*  
cells:

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SOC medium: 20 g of Bacto tryptone, 5 g of Bacto yeast extract, 0.5 g of NaCl, 0.2 g of KCl ad 1 l ddH<sub>2</sub>O, autoclave. Before use, add: 0.5 ml of 1 M MgCl<sub>2</sub>/1 M MgSO<sub>4</sub> (sterile-filtered), 1 ml of 1 M glucose (sterile-filtered)

LB(Amp) agar plates: mix together 1 l of LB medium (without ampicillin) and 15 g of agar-agar, autoclave, cool to about 60°C and 1 ml of ampicillin solution (100 mg/ml). Procedure:

- 10 Mixture 1-5  $\mu$ l of ligation product or plasmid DNA (5-50 ng/ $\mu$ l)
- 50  $\mu$ l of competent cells
- 450  $\mu$ l of SOC medium
- . thaw competent cells on ice for 10 min
- 15 . add DNA to the competent cells
- . incubate on ice for 30 min
- . heat shock: 30 sec at 42°C (water bath)
- . place cells on ice for 2 min
- . add 450  $\mu$ l of prewarmed SOC medium
- 20 . incubate at 37°C and 220 rpm for 1 h
- . streak 100  $\mu$ l portions of the mixture onto a prewarmed LB(Amp) plate
- . incubate plates at 37°C overnight

### Example 3:

- 25 TOPO-TA-Cloning<sup>®</sup> and ligation
- TOPO-TA-Cloning<sup>®</sup> is a five-minute cloning method for PCR products amplified with Taq polymerase.
- The TOPO-TA-Cloning<sup>®</sup> kit (version C) supplied by Invitrogen was developed for direct cloning of PCR
- 30 products. The system makes use of the property of thermostable polymerases which attach a single deoxyadenosine at the 3' end of all duplex molecules in a PCR (3'-A overhang). It is possible with the aid of these 3'-A overhangs to link the PCR products directly
- 35 to a vector which has 3'-T overhangs. The kit provides the pCR<sup>®</sup>2.1-TOPO vector which was specifically developed for this purpose. The vector is 3.9 kb in size and has a lacZ gene for blue/white selection, and ampicillin- and kanamycin-resistant genes. The cloning

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site is flanked on both sides by a single EcoRI cleavage site.

Ligation mixture:

- 2  $\mu$ l of fresh PCR product (10 ng/ $\mu$ l)
- 5 1  $\mu$ l of pCR<sup>®</sup>-TOPO vector
- 2  $\mu$ l of sterile water
- 5  $\mu$ l total volume
- Carefully mix the mixture and incubate at RT for 5 min
- 10 Briefly centrifuge and place tube on ice
- Employ ligation products immediately in the One Shot<sup>™</sup> transformation

- 15 A 5  $\mu$ l mixture without PCR product and consisting only of vector and water is used as control.

The One-Shot<sup>™</sup> transformation was carried out by the following method:

- Add 2  $\mu$ l of 0.5 M  $\beta$ -mercaptoethanol to the 50  $\mu$ l of One Shot<sup>™</sup> TOP10 competent cells thawed on ice;
- 20 Add 2  $\mu$ l of the TOPO-TA-Cloning<sup>®</sup> ligation per vial of competent cells;
- Incubate on ice for 30 min
- Heat shock: 30 sec at 42°C;
- Cool on ice for 2 min;
- 25 Add 250  $\mu$ l of SOC medium (RT);
- Incubate the vials at 37°C and 220 rpm for 30 min;
- Streak 100  $\mu$ l of each transformation mixture onto LB(Amp) plates prewarmed to 37°C;
- Incubate plates at 37°C overnight;
- 30 Analyse the resulting transformands after minipreparation (3.2.2.1) with suitable enzymes in an analytical restriction digestion.

Example 4:

- 35 *Gene expression in E. coli cells*

The procedure is outlined as follows:

The plasmid is isolated from successfully sequenced clones and transformed into the expression strain W3110

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. A clone is picked from the transformation plate and used to prepare a 5 ml ON preculture

. The preculture is streaked onto an LB(Amp) plate, and clones from this plate are used to inoculate  
5 expressions to be carried out later

. 1 ml of the preculture is then used to inoculate the 50 ml main culture (ratio 1:50) and the OD<sub>600</sub> is determined (reference measurement with uninoculated LB(Amp) medium)

10 . The main culture (in a 200 ml Erlenmeyer flask) is incubated at 37°C and 220 rpm

. The OD<sub>600</sub> is determined every 30 min

. Once the OD reaches 0.5, the cells are induced with 10 µl of anhydrotetracycline (1 mg/ml) per 50 ml  
15 of cell suspension (f.c. 0.2 µg of anhydrotetracycline per ml of cell suspension), and the OD is again determined (0 value)

. The OD is determined every hour and growth is stopped 3 h after the time of induction

20 . 1 ml of thoroughly mixed bacterial suspension is placed in a tube and centrifuged at 6000 rpm for 5 min (less suspension may also be used if necessary)

. The supernatant is aspirated off and the pellet is homogenized in 100 µl of 1 x red. sample buffer;

25 . The homogenate is boiled for 5 min, cooled on ice and briefly centrifuged;

. 10 µl of sample are loaded into each well of an SDS gel and the electrophoresis (3.2.16) is carried out;

30 . The gel is stained by Coomassie blue staining and/or by the method of Example 5.

Cell disruption:

Cells from a 50 ml overnight culture are centrifuged at 3500 rpm and 4°C for 15 min. The resulting supernatant  
35 is poured away and the cells are resuspended in 40 ml of 100 mM Tris/HCl (pH 8.5). The suspended cells are disrupted using a French press in a 1 inch cylinder under 18,000 psi. This entails the cells being forced through a narrow orifice (< 1 mm) and subjected to a

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sudden fall in pressure. The cells burst due to the pressure difference on passing through the orifice. The structure of the cellular proteins is retained during this. To avoid proteolytic degradation of the required protein, a protease inhibitor should be added immediately after the cell disruption. For this purpose, 1 tablet of the EDTA-free Complete™ Protease-Inhibitor Cocktail (Roche) is added to each 40 ml of protein solution and dissolved at RT. The subsequent centrifugation at 6000 rpm for 20 minutes removes the cell detritus and large parts of DNA and RNA. The samples are then frozen at -20°C.

#### Example 5:

Activity staining of the GlcDH band in the SDS gel:  
The glucose dehydrogenase band can be specifically detected in the SDS gel using iodophenylnitrophenylphenyltetrazolium chloride (INT). This is possible only because the SDS treatment does not destroy the GlcDH activity.  
The GlcDH is detected by means of a colour reaction. This entails the hydrogen formed in the reaction being transferred to the tetrazolium salt INT, producing a violet formazan. Phenanzine methosulfate serves as electron transfer agent.

Preincubation buffer (0.1 M Tris/HCl, pH 7.5)

15.76 g of Tris/HCl

ad 1 l ddH<sub>2</sub>O, pH 7.5 with NaOH

Reaction buffer (0.08% INT, 0.005% phenanzine methosulfate, 0.065% NAD, 5% Glc in 0.1 M Tris/HCl (pH 7.5))

0.8 g of iodophenylnitrophenyltetrazolium chloride (INT)

0.05 g of methylphenazinium methosulfate (phenanzine methosulfate)

0.65 g of NAD

50 g of D-(+)-glucose monohydrate (Glc)



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ad 1 l 0.1 M Tris/HCl (pH 7.5)

Storage buffer for GlcDH:

26.5 g of EDTA

5 15 g of  $\text{Na}_2\text{HPO}_4$

ad 1 l, pH 7.0 (NaOH)

Sample preparation:

- . Dilute samples and markers in sample buffer.
- 10 . Boil in a water bath for 3 min and cool on ice, and centrifuge.

SDS gel electrophoresis by standard methods.

15 Activity staining:

- . Incubate SDS gel with fractionated protein bands in preincubation buffer at 37°C with gentle shaking for 5 min
- . Pour off buffer and cover with a sufficient amount
- 20 of reaction buffer (RT), and incubate at 37°C with gentle shaking (change buffer at least 1 x)
- . After incubation for about 30 min, the bands with GlcDH are stained red-violet.
- . Wash gel in preincubation buffer, photograph and
- 25 dry
- . If required, carry out a subsequent Coomassie staining and then dry the gel.

Example 6:

30

*Immunological detection using the ECL system (Western Exposure<sup>TM</sup> Chemiluminescent Detection System):*

- Proteins coupled to a His tag are detected indirectly using two antibodies. The first Ab employed is the
- 35 anti-<sup>RGS</sup>His antibody (QIAGEN) for detecting 6xHis-tagged proteins. The resulting antigen-antibody complex is then detected using the peroxidase (POD)-labelled AffiniPure goat anti-mouse IgG (H+L) antibody. After addition of the ECL substrate mixture, the bound

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peroxidase results in a chemiluminescent product which can be detected using a high performance chemiluminescence film.

Ponceau S solution (0.5% Ponceau S, 7.5% TCA)

- 5    1.25 g of Ponceau S  
     18.75 g of TCA  
     Make up to 250 ml with double-distilled water.

10x PBS buffer pH 7.4

- 10    14.98 g of disodium hydrogen phosphate x 2 H<sub>2</sub>O  
     2.13 g of potassium dihydrogen phosphate  
     87.66 g of sodium chloride  
     Make up to 1 l, check that pH is 7.4.  
     The 1x concentration of the buffer is employed.

15

Biometra blot buffer

- 25 mM    Tris  
     150 mM   Glycine  
     10%      Methanol

20

Blocking reagent

- 5%      Skimmed milk powder  
     Dissolve in 1x PBS buffer.

25

Washing buffer

- 0.1%    Nonidet<sup>TM</sup> P-40 (Sigma)  
     Dissolve in 1x PBS buffer

The detection was carried out as follows:

- .      Cut a PVDF membrane (Immobilon P, Millipore) and  
30    6x blotting filter paper to the size of the gel  
     .      Equilibrate the PVDF membrane for 15 sec in  
         methanol and then in Biometra blot buffer, and apply  
         the same procedure to the SDS gel and the filter papers  
     .      Blot construction: assemble 3 layers of filter  
35    paper, membrane, gel, 3 layers of filter paper in the  
         blot chamber (air bubbles between the layers must be  
         expelled otherwise no protein transfer takes place at  
         these points)

     .      Blotting: 1-1.5 mA/cm<sup>2</sup> of gel for 1 h

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Check of protein transfer:

After the blotting, the protein transfer to the PVDF membrane is checked by staining with Ponceau S: incubate the membrane with 0.5% Ponceau S solution in a dish with gentle shaking for at least 2 min. Pour off dye (reusable) and destain the membrane under running deionized water. In this case, only strong protein bands are stained. The molecular weight marker is marked with a ballpoint pen.

10 Development of the blot:

All incubations should be carried out in a dish on a Celloshaker and in a roller cabinet in 50 ml Falcon tubes since the membrane must never dry out during the following steps.

15 (1) Saturation

30 min at 37°C in a roller cabinet with PBS/5% skimmed milk powder

(2) 1<sup>st</sup> antibody: incubate diluted 1:2000 in PBS/5% skimmed milk powder (volume about 7 ml/membrane) at 37°C for 1 h

20 (3) Washing: Wash membrane copiously with washing solution PBS/0.1% NP-40 wash for 3 x 5 min

(4) POD-labelled Ab: incubate diluted 1:1000 in PBS/5% skimmed milk powder (new tube) at 37°C for 1 h

25 (5) Washing: Wash membrane copiously with washing solution PBS/0.1% NP-40 wash for 3 x 5 min

(6) Development: Swirl membrane thoroughly (do not allow to dry) and place on a plastic sheet, cover completely with ECL developer solution (Amersham) for 1 min, swirl membrane and place in a doubled sheet, lay polaroid Hyperfilm on top and develop

30

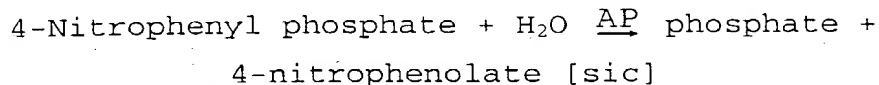
Example 7:

Tridegin detection by inhibition of factor XIIIa  
35 (Method of Finney et al., 1997, modified according to the invention):

In place of the natural substrate of factor XIIIa, namely amino-containing side chains of amino acids, synthetic amines are also incorporated into suitable

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protein substrates. These synthetic amines have intramolecular markers which make detection possible. The amine incorporation test is a solid-phase test. The titre plates are coated with casein. The substrate  
 5 biotinamidopentylamine is incorporated into this casein by factor XIIIa. The casein-biotinamidopentylamine product can be detected by the streptavidin-alkaline phosphatase fusion protein (strep/AP). This sandwich can take place [sic] by detecting the phosphatase  
 10 activity using p-nitrophenyl phosphate. This involves the following reaction:



The formation of 4-nitrophenolate [sic] is determined  
 15 by photometry at 405 nm and is directly proportional to the AP activity. The high-affinity interaction of biotin and streptavidin means that the phosphatase activity is likewise proportional to the factor XIIIa activity, that is to say a stronger absorption (yellow  
 20 coloration) means a higher factor XIIIa activity (Janowski, 1997). EDTA is a very nonspecific inhibitor of factor XIIIa, whose cofactor  $\text{Ca}^{2+}$  is bound by EDTA in a chelate complex. For this reason, the protein samples used must not contain any EDTA and were pretreated with  
 25 an EDTA-free protease inhibitor cocktail (Boehringer).

Washing buffer: 100 mM Tris/HCl, pH 8.5

Solution A: Dissolve 0.5% skimmed milk powder in washing buffer

Solution B: Dissolve 0.5 mM biotinamidopentylamine, 10 mM DTT,  
 30 5 mM  $\text{CaCl}_2$  in washing buffer

Solution C: Dissolve 200 mM EDTA in washing buffer

Solution D: Dissolve 1.7  $\mu\text{g/ml}$  of  
 35 streptavidin-alkaline phosphatase in solution A

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Solution E: Dissolve 0.01% (w/v) Triton X-100 in washing buffer

Solution F: Dissolve 1 mg/ml p-nitrophenyl phosphate; 5mM MgCl<sub>2</sub> in washing buffer

5

Coating:

. Distribute 200 µl of solution A in each well on a titre plate, depending on the number of samples  
Shake at 37°C for 30 min (Thermoshaker)

10 Washing:

. Wash twice with 300 µl of washing buffer per well

Incorporation reaction:

. Distribute 10-150 µl of sample per well and add 5 µl of factor XIIIa per well and 200 µl of solution B per well

15

Shake at 37°C for 30 min

Stopping:

. Wash twice with 300 µl of solution C (factor XIIIa inhibition) per well

20

. Wash twice with 300 µl of washing buffer per well

Strep/Ap binding (specific):

. Add 250 µl of solution D per well

. Incubate at RT for 60 min

Washing:

25

. Wash with 300 µl of solution E per well (detaches the proteins which are not covalently bonded)

. Wash 4 times with 300 µl of washing buffer per well

Substrate:

30

. Add 50 µl of solution F per well + 200 µl of washing buffer per well

. Incubate at RT for 30 min

Measure with computer-assisted evaluation in a microtitre plate reader at 405 nm

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EXAMPLE 8: Sensitivity of GlcDH detection

The stated amount of purified GlcDH was put on an SDS gel. After the run, the SDS gel was incubated in preincubation buffer at 37°C for 5 minutes. The buffer

- 40 -

was discarded and the gel was shaken in reaction buffer at 37°C. In a further step the gel was stained with Coomassie blue.

Reaction buffer for 1 litre:

- 5 0.1M Tris/HCL, pH 7.5
  - 0.5M NaCl
  - 0.2% Triton X-100
  - 0.8 g of iodophenylnitrophenyltetrazolium chloride
  - 0.05 g of methylphenazinium methosulfate
  - 10 0.65 g of NAD
  - 50 g of D-(+)-glucose monohydrate
- Preincubation buffer:
- 0.1M Tris/HCl, pH 7.5
  - 0.5M NaCl

1. Recombinant fusion protein consisting of at least a first and second amino acid sequence, characterized in that the first sequence has the biological activity of glucose dehydrogenase.
2. Recombinant fusion protein according to Claim 1, characterized in that the second sequence is any recombinant protein/polypeptide X or represents parts thereof.
3. Recombinant fusion protein according to Claim 2, characterized in that it may additionally have at least one other recognition sequence ("tag sequence") suitable for detection.
4. DNA, characterized in that it codes for a fusion protein according to Claims 1-3.
5. Expression vector, characterized in that it comprises a DNA according to Claim 4.
6. Host cell for expressing recombinant proteins/polypeptides, characterized in that it comprises an expression vector according to Claim 5.
7. Use of glucose dehydrogenase as detector protein for any recombinant protein/polypeptide X in a fusion protein according to Claims 1 to 3.
8. Use of glucose dehydrogenase in a detection system for the expression of a recombinant protein/polypeptide X as constituent of a fusion protein according to Claims 1 to 3.





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is employed to detect the enzymic activity of glucose dehydrogenase.

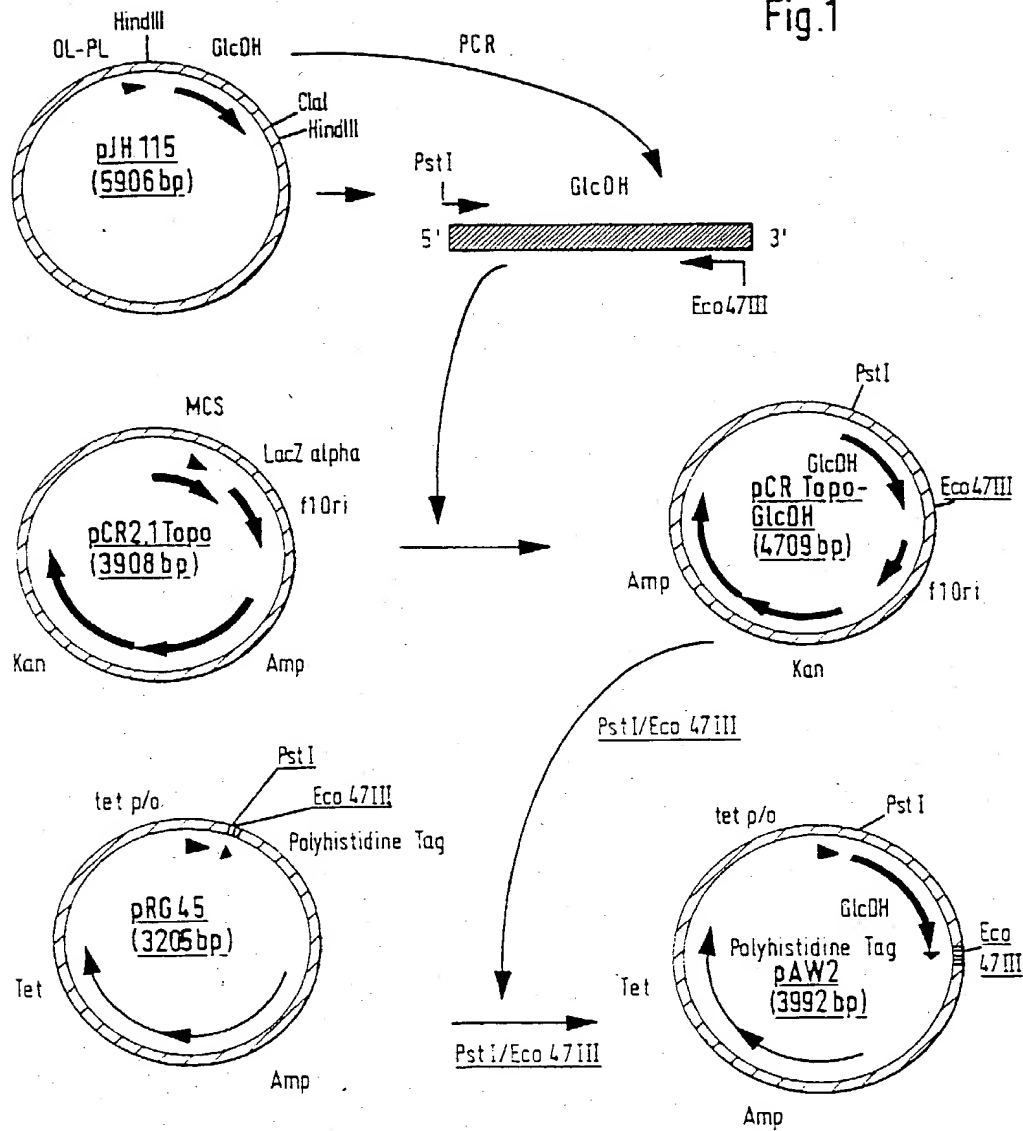
- 5 16. Method according to Claim 15, characterized in that iodophenylnitrophenyl-phenyltetrazolium salt (INT) or nitro blue tetrazolium salt (NBT) is employed as tetrazolium salt.
- 10 17. Method according to Claims 13 to 16, characterized in that the specific staining of the glucose dehydrogenase is followed by a general protein staining.

**PCT**WELTORGANISATION FÜR GEISTIGES EIGENTUM  
Internationales BüroINTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE  
INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

<b>(51) Internationale Patentklassifikation <sup>7</sup> :</b>  <b>C07K 14/00</b>	<b>A2</b>	<b>(11) Internationale Veröffentlichungsnummer:</b> <b>WO 00/49039</b>  <b>(43) Internationales Veröffentlichungsdatum:</b> 24. August 2000 (24.08.00)
<b>(21) Internationales Aktenzeichen:</b> PCT/EP00/00978 <b>(22) Internationales Anmeldedatum:</b> 8. Februar 2000 (08.02.00) <b>(30) Prioritätsdaten:</b> 199 06 920.4      19. Februar 1999 (19.02.99)      DE <b>(71) Anmelder (für alle Bestimmungsstaaten ausser US):</b> MERCK PATENT GMBH [DE/DE]; Frankfurter Strasse 250, D-64293 Darmstadt (DE). <b>(72) Erfinder; und</b> <b>(75) Erfinder/Anmelder (nur für US):</b> LINXWEILER, Winfried [DE/DE]; Bahnhofstrasse 48, D-64823 Gross-Umstadt (DE). BURGER, Christa [DE/DE]; Carsonweg 23, D-64289 Darmstadt (DE). PÖSCHKE, Oliver [DE/DE]; Paracelsusweg 7, D-65203 Wiesbaden (DE). HOFMANN, Uwe [DE/DE]; Hähnleinerstr. 42, D-64665 Alsbach (DE). WOLF, Andrea [DE/DE]; Lahrer Str. 15a, D-68239 Mannheim (DE). <b>(74) Gemeinsamer Vertreter:</b> MERCK PATENT GMBH; D-64271 Darmstadt (DE).		<b>(81) Bestimmungsstaaten:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Veröffentlicht</b> <i>Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.</i>
<b>(54) Title:</b> GLUCOSE DEHYDROGENASE FUSION PROTEINS AND THEIR UTILIZATION IN EXPRESSION SYSTEMS <b>(54) Bezeichnung:</b> GLUCOSE-DEHYDROGENASE-FUSIONSPROTEINE UND IHRE VERWENDUNG IN EXPRESSIONSSYSTEMEN <b>(57) Abstract</b> <p>The invention relates to novel recombinant fusion proteins containing a protein sequence having the biological activity of glucose dehydrogenase as one of its constituents and to their utilization for simple and efficient detection of any type of proteins/polypeptides in SDS-Page gels and for quick optimization of expression systems that can express the above-mentioned proteins/polypeptides.</p> <b>(57) Zusammenfassung</b> <p>Die Erfindung betrifft neue rekombinante Fusionsproteine, welche als ein Bestandteil eine Proteinsequenz mit der biologischen Aktivität von Glucose-Dehydrogenase enthalten sowie ihre Verwendung zum einfachen und effizienten Nachweis von beliebigen Proteinen/Polypeptiden in SDS-PAGE-Gelen und zur raschen Optimierung von Expressionssystemen, welche besagte Proteine/Polypeptide zu exprimieren in der Lage sind.</p>		

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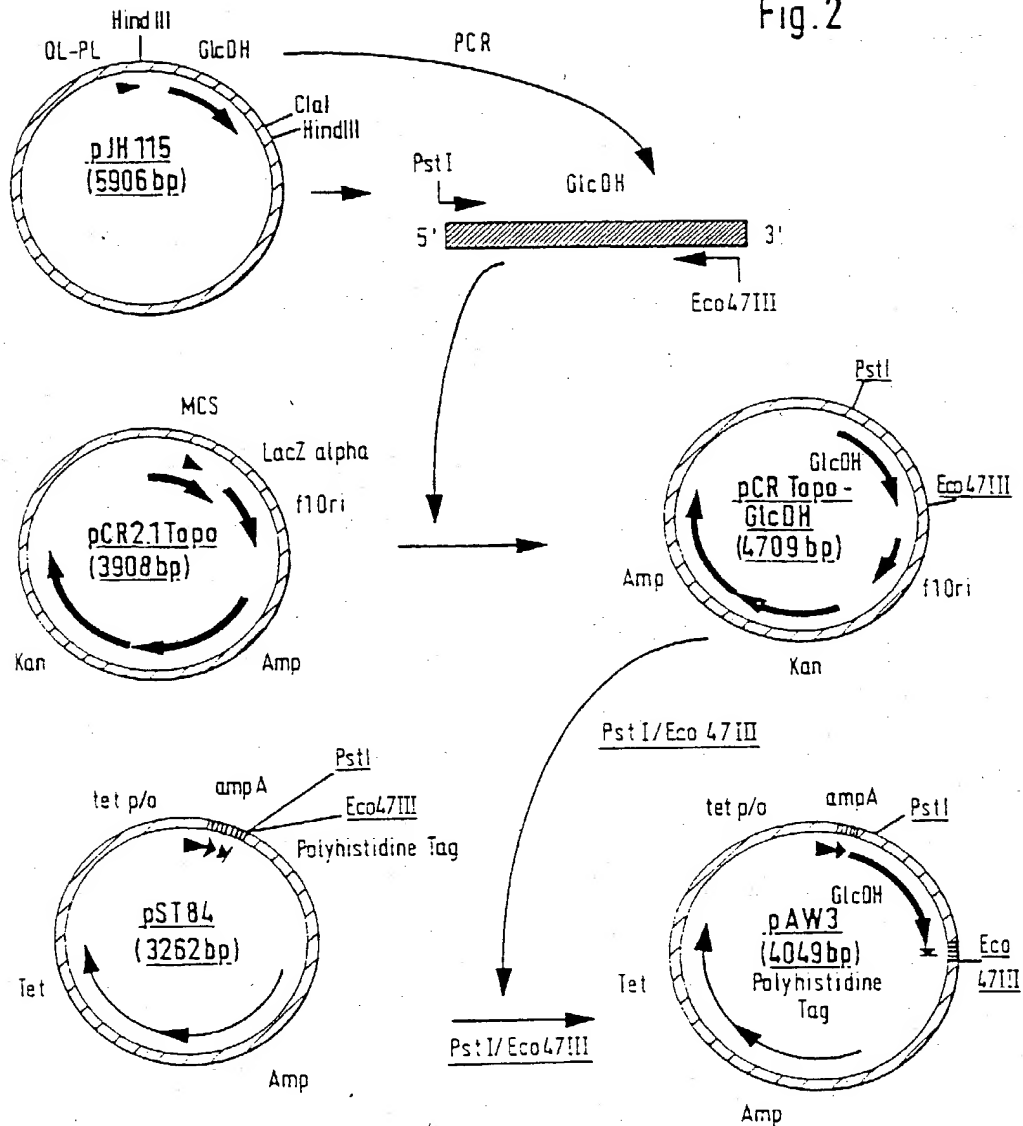
Fig.1



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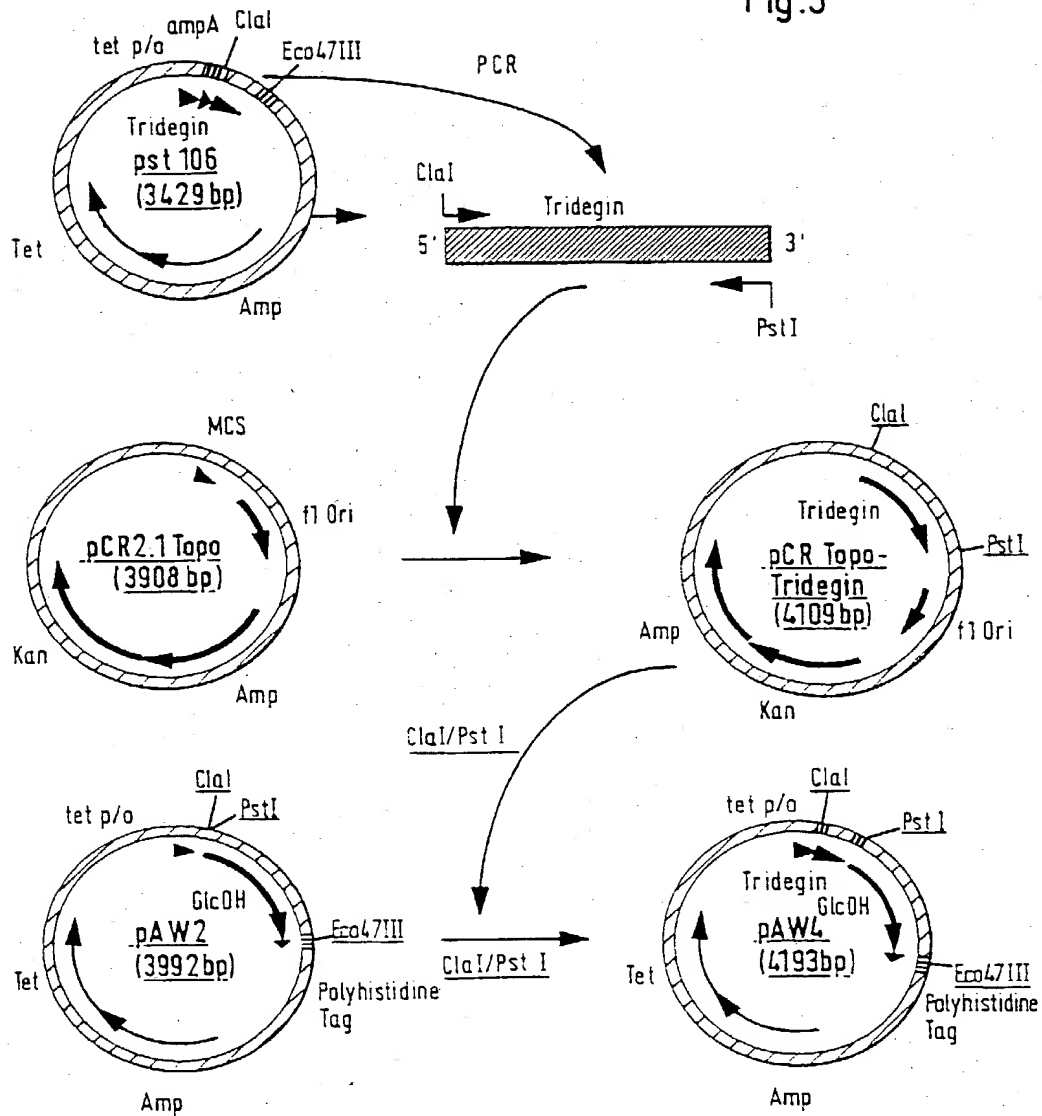
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Fig. 2



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Fig.3



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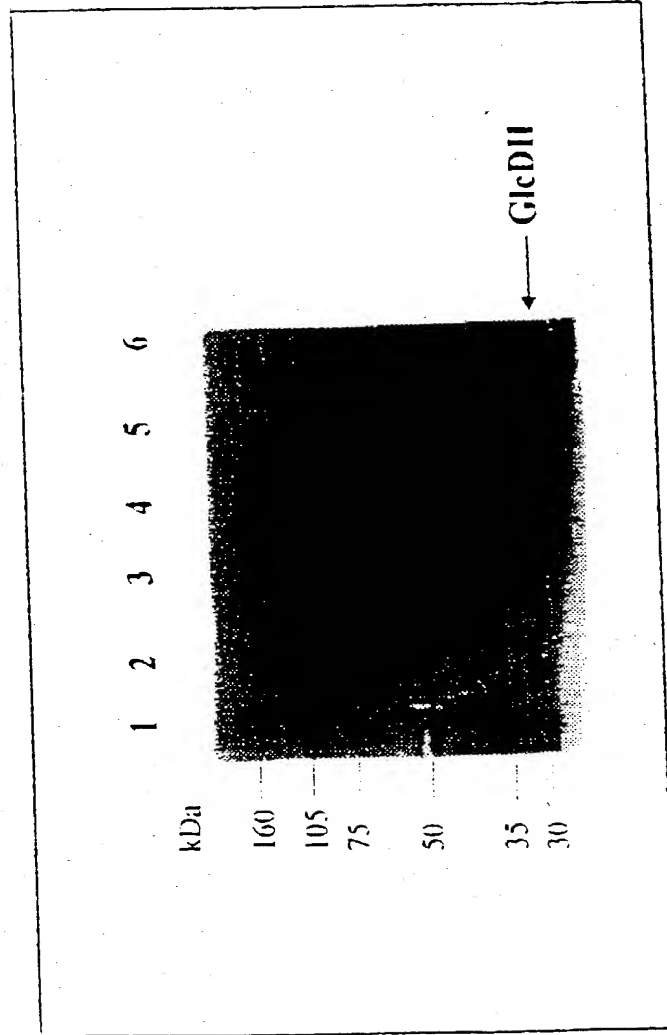


Fig. 4

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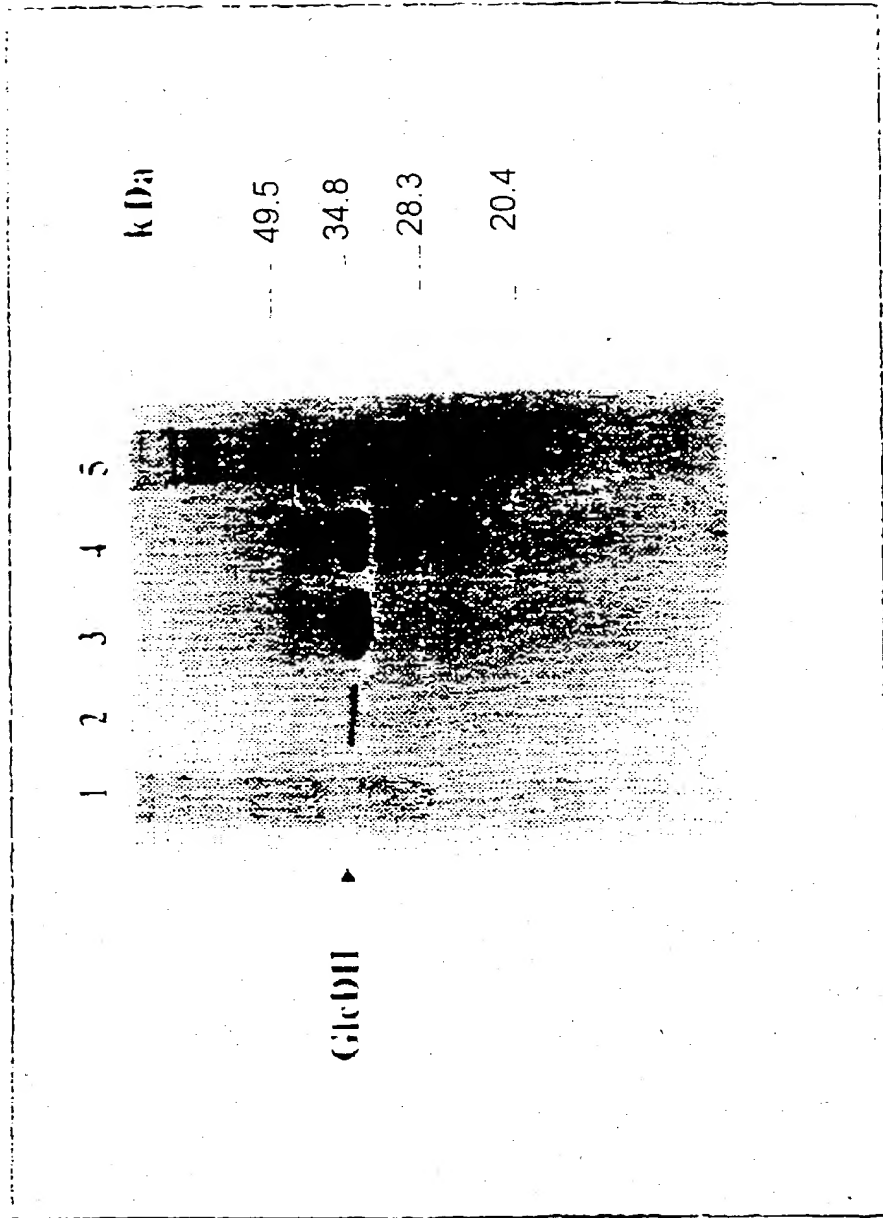
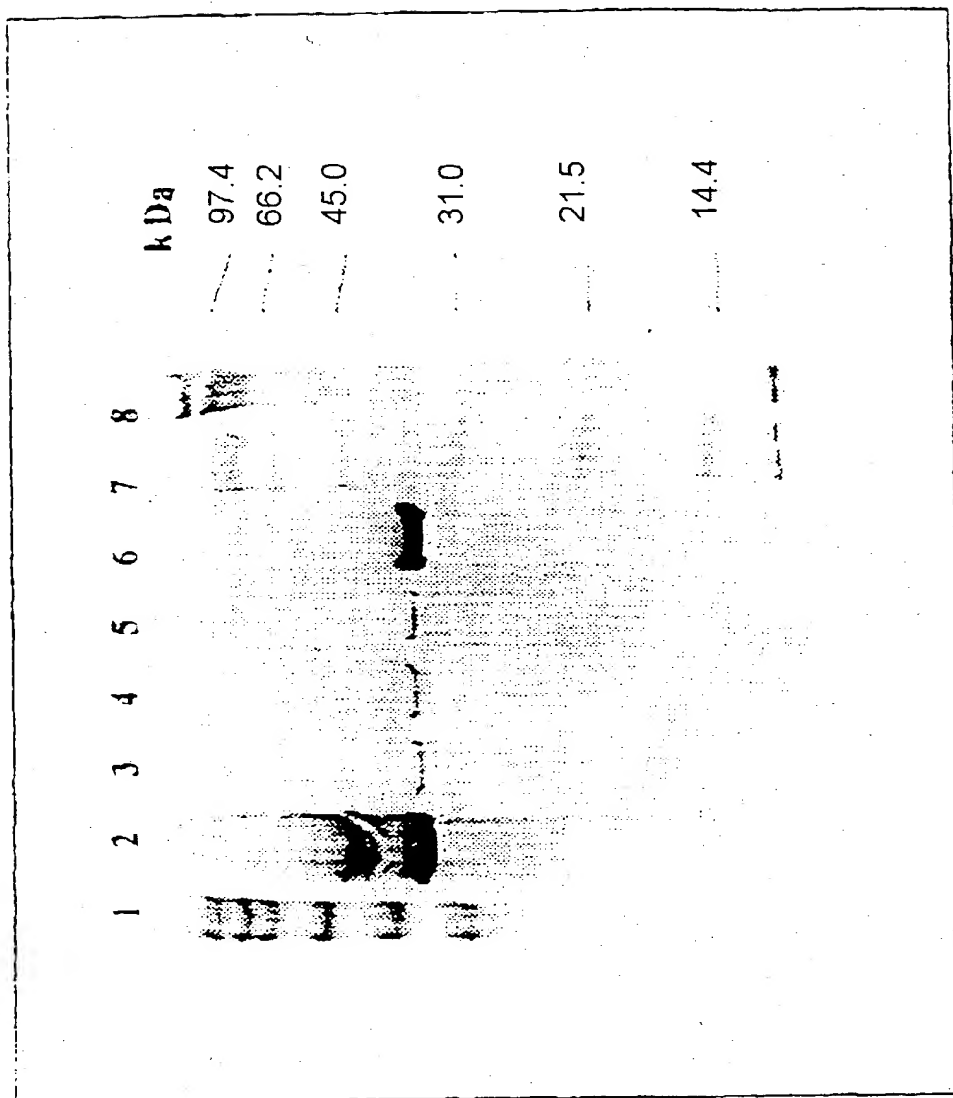


Fig. 5

Fig. 6



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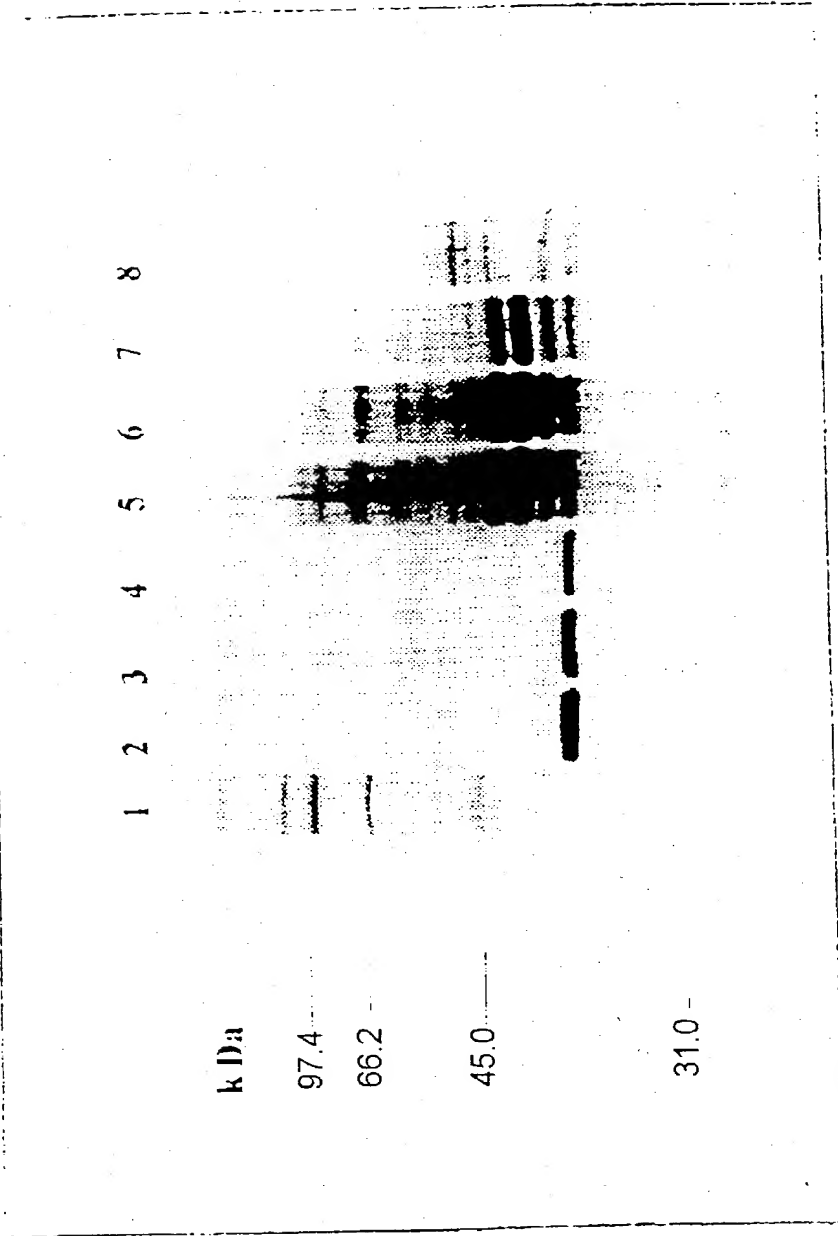
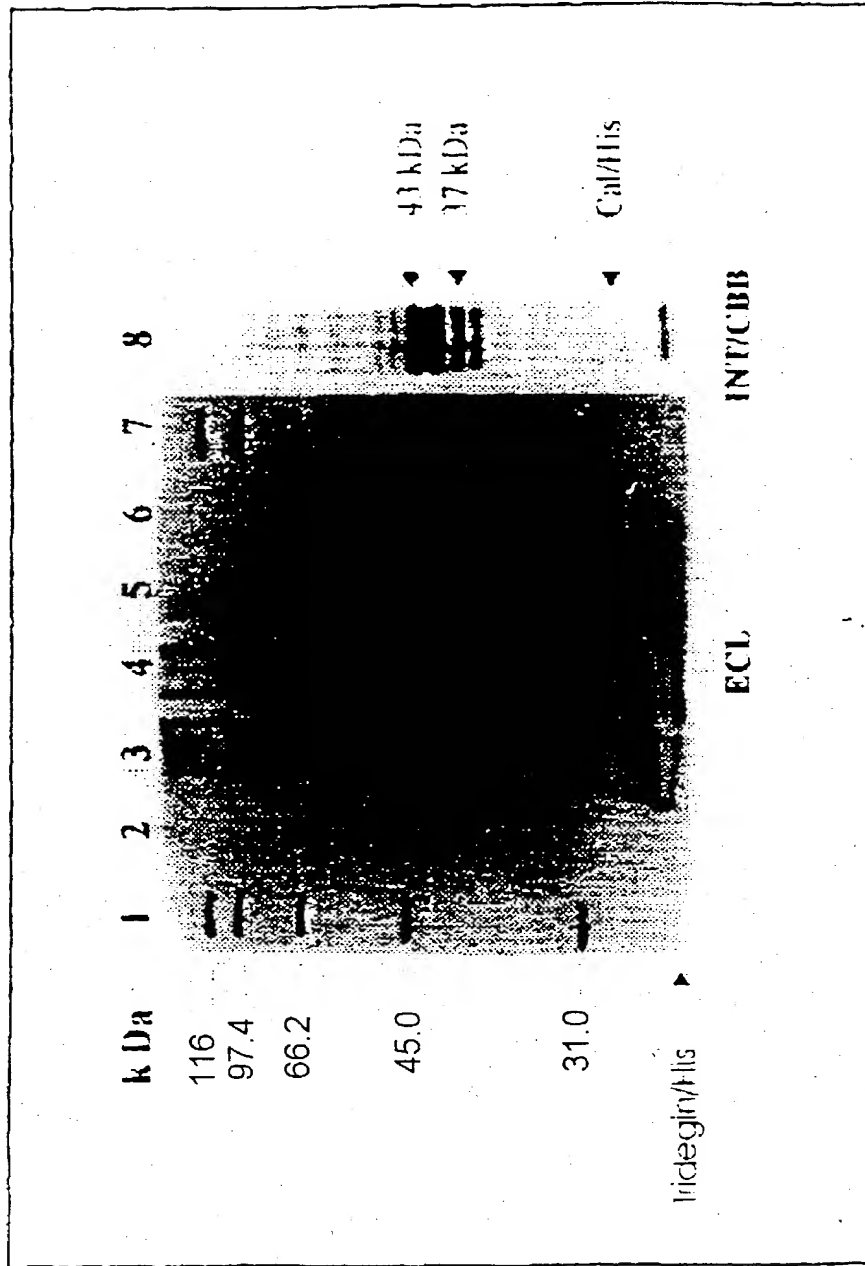


Fig. 7

Fig. 8



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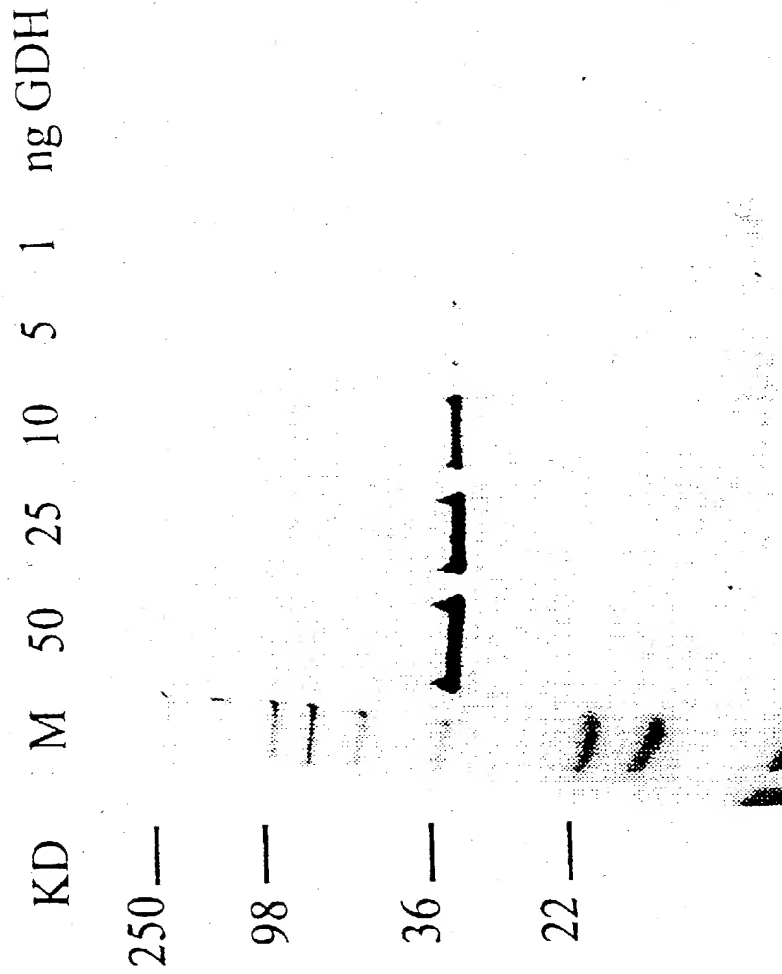


Fig. 9

Docket No.  
MERCK

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on \_\_\_\_\_ as United States Application No. or PCT International Application Number \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
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(Number)	(Country)	(Day/Month/Year Filed)	

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Sixth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

SEQUENCE LISTING

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<211> 272

<212> PRT

<213> Bacillus megaterium (GlcDH-polytag fusion protein)

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 20 25 30

Lys Val Val Ile Asn Tyr Tyr Asn Asn Glu Glu Glu Ala Leu Asp Ala  
 35 40 45

Lys Lys Glu Val Glu Glu Ala Gly Gly Gln Ala Ile Ile Val Gln Gly  
 50 55 60

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Lys Glu Phe Gly Thr Leu Asp Val Met Ile Asn Asn Ala Gly Val Glu  
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<210> 3
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<212> DNA
<213> Bacillus megaterium + Heamenteria ghiliani fusion gene

<220>
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<223> Plasmid PAW4

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<220>
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agataacgag ggcaatcgat atg aaa cta ttg cct tgc aaa gaa tgg cat caa 173  
Met Lys Leu Leu Pro Cys Lys Glu Trp His Gln  
1 5 10  
ggt att cct aac cct agg tgc tgg tgt ggg gct gat cta gaa tgc gca 221  
Gly Ile Pro Asn Pro Arg Cys Trp Cys Gly Ala Asp Leu Glu Cys Ala  
15 20 25  
caa gac caa tac tgt gcc ttc ata cct caa tgt aga cca aga tca gaa 269  
Gln Asp Gln Tyr Cys Ala Phe Ile Pro Gln Cys Arg Pro Arg Ser Glu  
30 35 40  
ctg att aaa cct atg gat gat ata tac caa aga cca gtc gag ttt cca 317  
Leu Ile Lys Pro Met Asp Asp Ile Tyr Gln Arg Pro Val Glu Phe Pro  
45 50 55  
aac ctt cca tta aaa cct agg gag gaa agcgcctatga gaggatcgca 364  
Asn Leu Pro Leu Lys Pro Arg Glu Glu  
60 65  
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Met Tyr Thr Asp Leu Lys Asp Lys Val Val  
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Phe Gly Gln Glu Glu Ala Lys Val Val Ile Asn Tyr Tyr Asn Asn Glu  
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gaa gaa gct cta gat gcg aaa aaa gaa gta gaa gaa gca gcc gga caa 560  
Glu Glu Ala Leu Asp Ala Lys Lys Glu Val Glu Glu Ala Gly Gly Gln  
115 120 125  
gca atc atc gtt caa gcc gat gta aca aaa gaa gaa gac gtt gta aat 608  
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Ala Phe Ile Pro Gln Cys Arg Pro Arg Ser Glu Leu Ile Lys Pro Met  
 35 40 45

Asp Asp Ile Tyr Gln Arg Pro Val Glu Phe Pro Asn Leu Pro Leu Lys  
 50 55 60

Pro Arg Glu Glu Met Tyr Thr Asp Leu Lys Asp Lys Val Val  
 65 70 75

Val Ile Thr Gly Gly Ser Thr Gly Leu Gly Arg Ala Met Ala Val Arg  
 80 85 90

Phe Gly Gln Glu Glu Ala Lys Val Val Ile Asn Tyr Tyr Asn Asn Glu  
 95 100 105 110

Glu Glu Ala Leu Asp Ala Lys Lys Glu Val Glu Glu Ala Gly Gly Gln  
 115 120 125

Ala Ile Ile Val Gln Gly Asp Val Thr Lys Glu Glu Asp Val Val Asn  
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Leu Val Gln Thr Ala Ile Lys Glu Phe Gly Thr Leu Asp Val Met Ile  
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Asn Asn Ala Gly Val Glu Asn Pro Val Pro Ser His Glu Leu Ser Leu  
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Asp Asn Trp Asn Lys Val Ile Asp Thr Asn Leu Thr Gly Ala Phe Leu  
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Gly Ser Arg Glu Ala Ile Lys Tyr Phe Val Glu Asn Asp Ile Lys Gly  
 195 200 205

10

Asn Val Ile Asn Met Ser Ser Val His Glu Met Ile Pro Trp Pro Leu  
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 Phe Val His Tyr Ala Ala Ser Lys Gly Gly Met Lys Leu Met Thr Glu  
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 Thr Leu Ala Leu Glu Tyr Ala Pro Lys Gly Ile Arg Val Asn Asn Ile  
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 Pro Glu Gln Arg Ala Asp Val Glu Ser Met Ile Pro Met Gly Tyr Ile  
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 Gly Lys Pro Glu Glu Val Ala Ala Val Ala Ala Phe Leu Ala Ser Ser  
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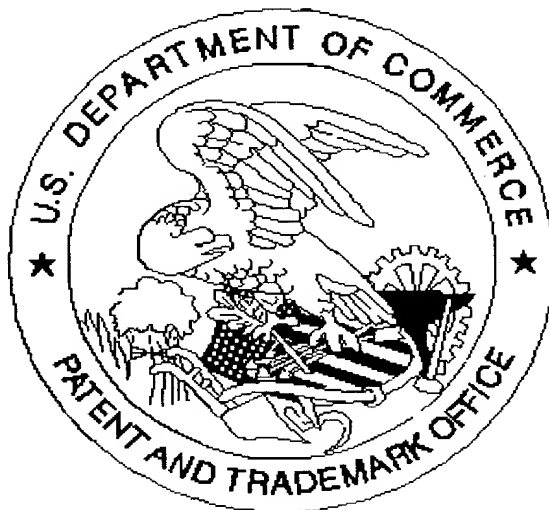
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